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Environmental Enrichment Rescues Plasticity of Serotonergic Neurons

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Tiivistelmä – Referat – Abstract

Neurotrophin, Brain-derived neurotrophic factor (BDNF) and its cognate receptor Tropomyosin receptor kinase B (TrkB), have been concomitantly linked with neuronal plasticity as well as antidepressant mechanism of action. Adult hippocampal neurogenesis involves proliferation and survival of new-born neurons and has been related to antidepressant mechanisms and cognitive improvement. Environmental enrichment (EE) enhances adult hippocampal neurogenesis (AHN) and induces anxiolytic-like effects. This study postulates that EE-living conditions could restore the abnormal serotonergic modulation on AHN of our transgenic mice. In this study, a transgenic mouse line wherein TrkB receptor is compromised from serotonergic neurons and AHN found to be impaired was used. To assess the behavioural effects and the changes in learning and memory tasks produced by 10-weeks of EE, a behavioural battery test was performed. Our results suggested anxiolytic-like effects from EE in the transgenic mice. Likewise, cognitive improvements were also observed in both control and transgenic mice promoted by EE. Moreover, hyperactivity observed in transgenic mice in standard conditions could be rescued, and no phenotypical differences were observed between control and transgenic mice subjected to EE. To further study the effects of EE on AHN, cellular proliferation and survival were studied through the incorporation of BrdU. The results indicate that the abnormal serotonergic regulation of AHN was rescued upon EE-living conditions. Moreover, molecular methods used to measure the alteration of gene expression revealed significant upregulation of genes related to neuronal plasticity and epigenetic modifications. Altogether, these results suggest EE promotes the neuronal plasticity, rescues the impaired regulation of AHN and modulates the genetic expression of the transgenic mice. Findings from this study could provide new insights regarding novel targets that could modulate adult brain plasticity.

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To Maria, for our friendship.

TABLE OF CONTENTS

1. **INTRODUCTION** 7

1.1 **NEUROTROPHIC FACTORS** 7
1.2 **ADULT HIPPOCAMPAL NEUROGENESIS** 8
1.2.1 ADULT HIPPOCAMPAL NEUROGENESIS AND BDNF 11
1.2.2 REGULATION OF ADULT HIPPOCAMPAL NEUROGENESIS 12
1.3 **SEROTONERGIC SYSTEM** 13
1.3.1 SEROTONIN AND ADULT HIPPOCAMPAL NEUROGENESIS 15
1.3.2 SEROTONIN AND BDNF 16
1.4 **ENVIRONMENTAL ENRICHMENT** 17
1.5 **CONDITIONAL KNOCKOUT ANIMALS** 19

2. **HYPOTHESIS AND OBJECTIVES** 20

3. **MATERIALS AND METHODS** 21

3.1 **EXPERIMENTAL ANIMAL MODEL** 21
3.1.1 ANIMAL LICENSE 23
3.1.2 CRE ACTIVATION USING TAMOXIFEN 23
3.2 **ANIMAL HOUSING CONDITIONS** 24
3.2.1 STANDARD HOUSING CONDITION 24
3.2.2 ENRICHED ENVIRONMENT HOUSING CONDITION 24
3.3 **BEHAVIOURAL BATTERY** 25
3.3.1 LIGHT-DARK BOX TEST 25
3.3.2 MARBLE BURYING TEST 26
3.3.3 NOVEL OBJECT RECOGNITION TEST 28
3.3.4 CUED AND CONTEXTUAL FEAR CONDITIONING TEST 30
3.3.5 PRE-PULSE INHIBITION OF STARTLE RESPONSE TEST 32
3.3.6 FORCED SWIMMING TEST 34
3.4 **SAMPLE COLLECTION** 35
3.4.1 BRAIN DISSECTION 36
3.4.2 PREFRONTAL CORTEX DISSECTION 37
3.4.3 HIPPOCAMPUS AND CORTEX DISSECTION 39
3.4.4 MIDBRAIN DISSECTION 41
3.5 **MOLECULAR PROCEDURES** 41
3.5.1 BROMODEOXYURIDINE TREATMENT 41
3.5.2 REAL-TIME REVERSE TRANSCRIPTION QUANTITATIVE POLYMERASE-CHAIN REACTION (RT-QPCR) 45
3.6 **STATISTICAL ANALYSIS** 47
3.7 **REAGENTS** 48

4. **RESULTS** 52

4.1 **ENVIRONMENTAL ENRICHMENT PROMOTES MEMORY ENHANCEMENT AND RESCUES THE HYPERACTIVE-LIKE PHENOTYPE OF TRANSGENIC MICE** 52

4.2 **ENRICHED ENVIRONMENT PROMOTES ANXIOLYTIC AND ANTIDEPRESSANT-LIKE EFFECTS** 53
4.3 **ENVIRONMENTAL ENRICHMENT INCREASES THE FREEZING BEHAVIOUR AND IMPAIRS FEAR EXTINCTION** 58
4.4 **ENVIRONMENTAL ENRICHMENT AMELIORATES THE ABNORMAL REGULATION OF ADULT HIPPOCAMPAL NEUROGENESIS OF TRANSGENIC MICE** 59
4.5 **ENVIRONMENTAL ENRICHMENT AFFECTS EPIGENETIC MODIFICATION OBSERVED IN THE HIPPOCAMPUS** 60

5. **DISCUSSION** 62

6. **CONCLUSIONS AND FUTURE PERSPECTIVES** 66

7. **ACKNOWLEDGMENTS** 67

8. **REFERENCES** 68

ABBREVIATIONS

5-HT	Serotonin
ACh	Acetylcholine
AHN	Adult hippocampal neurogenesis
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
cDNA	Complementary DNA
CO_2	Carbon dioxide
CREB	cAMP response element-binding protein
$CreER^{T2}$	Tamoxifen-dependent Cre recombinase
CS	Conditioned stimulus
DCX	Doublecortin
DG	Dentate gyrus
EE	Environmental enrichment
FC	Cued and Contextual Fear Conditioning Test
FST	Forced swim test
GCs	Granule cells
HCl	Hydrochloric acid
HDAC	Histone deacetylases
HP	Hippocampus
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
LD	Light-dark test
LTD	Long-term depression
LTP	Long-term potentiation
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MB	Marble burying
miRNAs	Micro RNAs
MSK1	Mitogen/stress-activated protein kinase-1
NFDM	Non-fat dry milk
NGF	Nerve growth factor
NOR	Novel object recognition test
NPCs	Neural progenitor cells
NSCs	Neural stem cells
NTs	Neurotrophins
OB	Olfactory bulb
$p75^{NRT}$	pan-neurotrophin receptor
PBS	Phosphate-buffered saline
PFC	Prefrontal cortex
PKA	Protein kinase A
PPI	Pre-Pulse Inhibition of Startle Response Test
PPS	Pre-pulse stimulus
RMS	Rostral migratory stream
SEM	Standard error of means
SERT	Serotonin transporter
SGZ	Subgranular zone
SH	Standard housing
SS	Startle stimulus
SSRI	Serotonin reuptake inhibitor
Tbr2	T-box brain protein 2
TBS	Tris-buffered saline
TPH2	Tryptophan hydroxylase 2

TrK	Tyrosine receptor kinase
TrkB	Tropomyosin receptor kinase B
US	Unconditioned stimulus
VMAT 2	Vesicular monoamine transporter 2

1. INTRODUCTION

1.1 Neurotrophic factors

The regulatory role of neurotrophins (NTs) in neuronal cell processes such as survival, differentiation, and maintenance of functions, has been well-established (Levi-Montalcini, 1987). After the discovery of the first neurotrophin, nerve growth factor (NGF) by Levi-Montalcini, Hamburger, and Cohen (1951), new growth factors have been included to the neurotrophin family, such as brain-derived neurotrophic factor (BDNF) (Barde, Edgar, and Thoenen 1982), NT-3 (Maisonpierre et al., 1990), and NT-4/5 (Ip et al., 1992). Moreover, exclusively in Zebrafish (*Danio rerio*), NT-6 and NT-7 were identified as new neurotrophin members (Götz et al. 1994; Nilsson et al. 1998). Beyond the structural similarities (Ibáñez, 1994), the cellular mechanisms triggered by NTs are mediated either by the high-affinity tyrosine receptor kinases (Trks/NTRKs) or by a low-affinity pan-neurotrophin receptor ($p75^{NTR}$) (Poo, 2001). The NTs exhibit their trophic actions by binding to their respective receptors, such as NGF with TrkA/NTRK1, BDNF and NT-4/5 with TrkB/NTRK2, while NT-3 shows a preference to TrkC/NTRK3 (Sendtner, 2005). Interestingly, pro-BDNF, the noncleaved precursor of BDNF, interacts preferentially with $p75^{NTR}$ (Chao and Bothwell, 2002).

The binding of NTs to Trk receptors initiates the activation and the dimerization of the receptors, which triggers the autophosphorylation of their tyrosine residues located in the cytoplasmic domain (Finkbeiner et al., 1997). The phosphorylation acts as a first step for the subsequent signal transduction, allowing the signalling cascades of downstream cytoplasmic effectors. This may culminate in the regulation of a broad-spectrum processes, such as morphogenetic modification (McAllister, Lo, and Katz, 1995), neuronal development (Lesser, Sherwood, and Lo, 1997), or synaptic transmission (Kang and Schuman, 1995). Concurrently, the cytoplasmic effectors, activated by the binding of NTs to its receptor, may promote long-term effects, which involve gene regulation (E. Li and Hristova 2006).

Besides the role of NTs as a regulatory factor in neuronal development, several studies have established the modulatory role of NTs, especially BDNF, in long-term plasticity in the adult nervous system (Poo, 2001). The induction of long-term potentiation (LTP) enhances mRNA levels of BDNF and TrkB, evidence that supports the hypothesis of

BDNF as a regulatory factor in synaptic plasticity mechanisms (Poo 2001; Nagappan and Lu 2005; Bramham et al. 1996). Interestingly, apoptosis, dendritic pruning and promotion of long-term depression (LTD) have been reported when $p75^{NTR}$ is activated by pro-BDNF (F. S. Lee and Chao 2002; Woo et al. 2005; Zagrebelsky 2005), indicating that neural plasticity requires a balanced combination of "catabolic" and "anabolic" processes (Lu, Pang, and Woo, 2005). Therefore, NTs and BDNF in particular, are the drivers of synaptic plasticity, which is considered as the pivotal process of brain plasticity, learning, memory and neurogenesis (Lamprecht and LeDoux 2004; Lee and Son 2009).

1.2 Adult hippocampal neurogenesis

The sequential developmental events that leads to the generation of new neurons in the adult brain is known as adult neurogenesis (Cameron, Woolley, McEwen, and Gould, 1993). Neurogenesis has been described in several brain regions of diverse vertebrates, such as bird, reptiles and fishes (Zupanc, 2001). Mammalian neurogenesis has been confirmed in confined areas, such as the subgranular zone (SGZ) of dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle whereby the adult stem cells migrate through rostral migratory stream (RMS) to the olfactory bulb (OB), where they differentiate into interneurons (Gage, 2000). Human neurogenesis, however, has been reported in the hippocampus and neocortex especially in the striatum (Eriksson et al. 1998; Ernst and Frisén 2015). Several contradictory findings along the line also exists (Kempermann, Kuhn, and Gage 2018; Sorrells et al. 2018). Additionally, the uncertainty remains about the characterization of human neurogenesis in the OB (Bergmann et al., 2012).

The proliferation of new functional granule cells (GCs) from neural stem cells (NSCs) after the amplification of intermediate progenitor cells and its functional integration into the existing neural circuits is known as adult hippocampal neurogenesis (AHN) (Van Praag et al., 2002). The neurogenic niche located in the SGZ of DG is composed of self-renewing and multipotent NSCs. These NSCs can proliferate and differentiate into diverse cell types, such as neurons and glia (**Figure 1A**) (Roy et al. 2000; Palmer, Willhoite, and Gage 2000). Two different NSCs have been reported in the neurogenic niche: radial glial cell - type 1, and non-radial type 2 cells, which give rise to intermediate progenitors, the neural progenitor cells (NPCs). These intermediate progenitors are

considered rapidly proliferative cells with a reduced progenies production (Hodge et al. 2008; Bonaguidi et al. 2012). During migration into the inner granule cell layer, the immature neurons start to differentiate into dentate granule cells, projecting dendritic arborization toward the molecular layer and axonal terminal through the hilus toward the CA3 **(Figure 1A)** (Zhao, 2006). Throughout the diverse developmental stages, the synaptic integration of the new-born neurons into the existing circuitry is critical. The NSCs and NPCs are tonically activated by ambient GABA originated from neighbouring parvalbumin basket cells, whereas the immature neurons receive GABAergic synaptic inputs, which gradually shift to glutamatergic inputs when the neuron acquires a mature-like stage (Bhattacharyya et al. 2008; Ge et al. 2006; Esposito 2005). Therefore, GABA and glutamate are considered local circuit factors in the regulation of AHN (Sierra et al. 2010; Tashiro et al. 2006; Schmidt-Hieber, Jones, and Bischofberger 2004). After approximately 8 weeks, the time needed for the new-born neurons to achieve the maturation stage, neurons exhibit similar electrophysiological and anatomical properties as mature neurons (Mongiat and Schinder, 2011). Upon fully integrated into the circuitry, the new granule cells become the excitatory principal neurons of the dentate gyrus (Toni et al., 2007). Integrating the inputs from the entorhinal cortex, granule cells emerge their axonal projection along the mossy fibre tract to CA3, providing excitatory inputs to the pyramidal cells CA3 (**Figure 1B**).

Exceptional advancements in the study of neurogenesis have been achieved through the development of techniques, such as the labelling of dividing cells with Bromodeoxyuridine (BrdU) and the characterization of specific proteins, known as markers, of the different developmental stages of neurogenesis. NSCs type 1 and type 2 characteristically express intermediate filament Nestin, (Filippov et al., 2003), the expression of helix-loop-helix factor Sox2 is characteristic of stem-cell-like cells (Steiner et al. 2006; D'Amour and Gage 2003). On the other hand, Type 2 cells express differentially Eomes (Tbr2), which is related to the suppression of *Sox2* and is crucial for the development of intermediate progenitors (Mussar et al., 2012). The exit of cell-cycle and post-mitotic maturation of the immature neurons can be labelled through the expression of Doublecortin (DCX) (Plümpe et al., 2006). When structural integration of the new-born neurons has been achieved, a change in their calcium-binding properties is observed. Calretinin, the protein involved in calcium signalling, is substituted by calbindin. This change in the calcium signalling proteins is used as a neural maturation

and survival marker (Brandt et al., 2003). The survival of the new-born cells, moreover, can be labelled through the expression of NeuN, a post mitotic marker expressed by the cell after the cell-cycle exit (Eriksson et al., 1998). Recently, epigenetic markers, such as histone deacetylases (HDAC) or small non-coding ribonucleic acids (miRNAs), are used as neurogenesis indicators due to their regulatory properties whereby the accessibility of DNA and histone is altered, shaping the cellular transcriptome landscape (Jawerka et al. 2010; Sun et al. 2011).

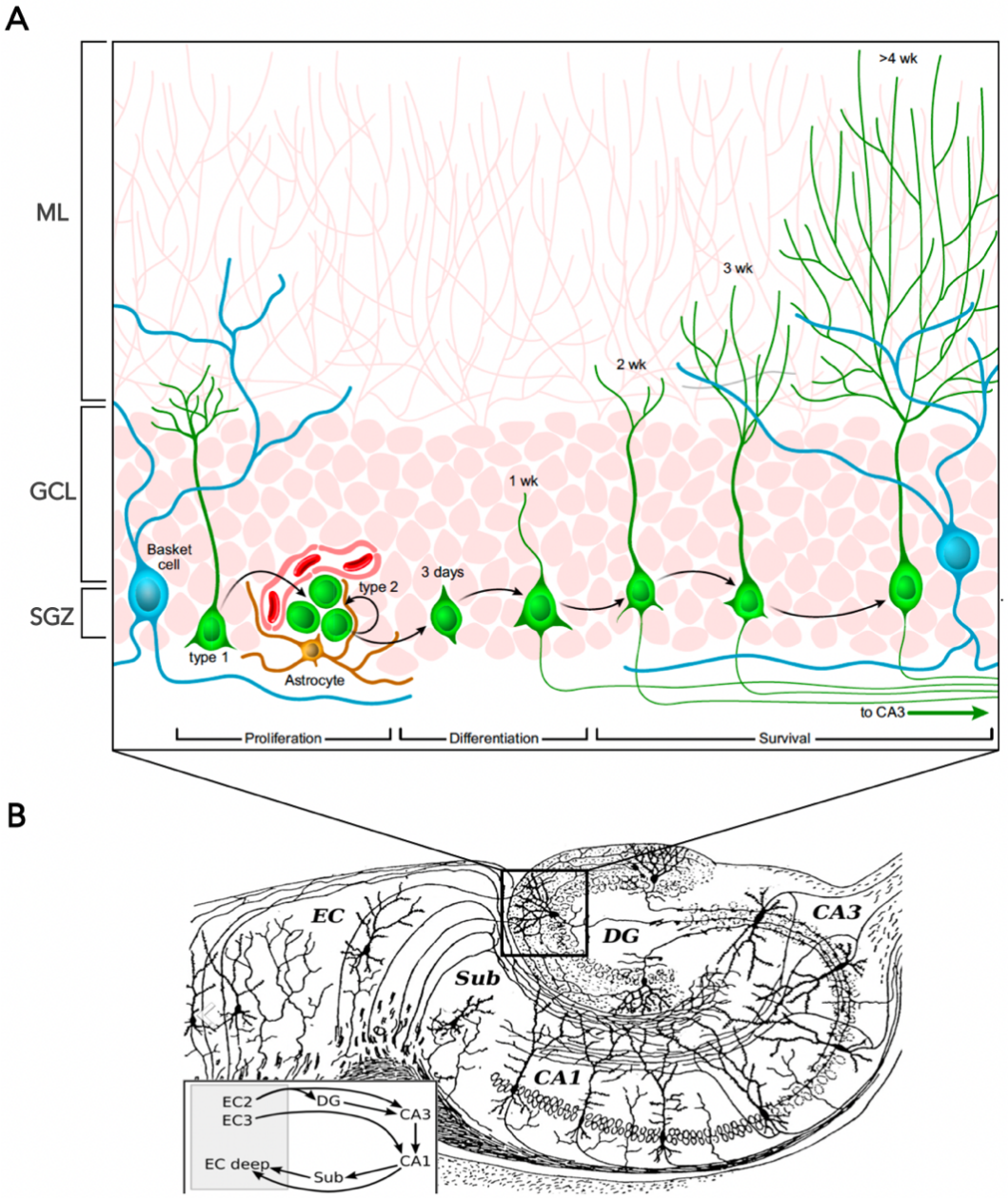


Figure 1 | Schematic illustration of the new-born hippocampal neurons development and the synaptic circuit anatomy of the hippocampus. A | The neural stem cells, formed by slowing diving cells type 1- radial glial cells-, and rapidly amplifying type 2 - neural progenitor cells. The neural progenitors start to differentiate over the two to three

weeks. By projecting dendritic arborizations to the molecular layer, the intermediate progenitors start to receive excitatory inputs around the second week. After 4-8 weeks, the new granule cells acquire the physiological and anatomical properties of a fully mature neuron, considered a new-born neuron. Adapted from (Aimone et al. 2014). **B|** Adapted schematic representation of the basic circuits of the hippocampus (Ramón y Cajal, 1909). ML: molecular layer; GCL: granule cell layer; SGZ: subgranular zone; DG: dentate gyrus; Sub: subiculum; EC: entorhinal cortex.

For several decades, adult neurogenesis has caught the attention of the neuroscientist community. Despite controversial studies (Sorrells et al. 2018; Boldrini et al. 2018), it has been accepted that new neurons in the hippocampus are essential for learning and memory (Squire 1992; Sahay and Hen 2007; Toda et al. 2018). The first evidence supporting this hypothesis was the correlation between neurogenesis and a better performance in specific hippocampus-dependent behavioural tasks (Shors et al., 2001). Subsequently, several findings have demonstrated that new-born neurons in the hippocampus have a predominant contribution to a wide range of spatial-related brain performances such us navigation learning, pattern discrimination, long-term memory or contextual fear conditioning (Sahay, Wilson, and Hen 2011; Aimone, Deng, and Gage 2011). Moreover, it has been suggested that neurogenesis is a requisite for particular antidepressant-induced behavioural responses (Sahay and Hen 2007; Antila et al. 2017), supported by the evidence that antidepressant treatments trigger neural plasticity (Castrén, 2005). While clear findings have suggested that AHN is enhanced by antidepressants (Jacobs, Van Praag, and Gage 2000; Sairanen 2005), it remains unclear whether antidepressant effects are the cause or effect mediating AHN depending on the underlying signaling pathways (Santarelli et al. 2003; Surget et al. 2008; David et al. 2009). Meanwhile, several studies have reported the association between aberrant neurogenesis and pathological conditions, such as epilepsy, neurodegenerative diseases and severe cognitive impairments (Cayre, Canoll, and Goldman 2009; Kron, Zhang, and Parent 2010).

1.2.1 Adult hippocampal neurogenesis and BDNF

The generation of functional neurons from NSCs essentially requires neurotrophic factors. Several studies have extensively demonstrated the role of neurotrophic factors, particularly BDNF, in the enhancement of proliferation of new-born hippocampal neurons (Greene et al. 2004; Scharfman et al. 2005; Tashiro et al. 2006; Li et al. 2008)).

Likewise, BDNF signaling has been implicated to the integration of new neurons into the hippocampal circuit and their successive survival (Babu, Ramirez-Rodriguez, Fabel, Bischofberger, and Kempermann, 2009). The phosphorylation of mitogen/stress-activated kinase 1 (MSK1) is enhanced by the binding of BDNF to TrkB (Rakhit, 2004). Upon MSK1 activation, mitogen-activated protein kinase (MAPK) signalling triggers the phosphorylation of transcription factor cAMP response element-binding protein (CREB), followed by the NF_kB pathway and subsequent regulation of immediate early genes (Arthur, 2008). In this regards, MSK1 has been related to the regulation of synaptic plasticity governed by BDNF (Karelina et al., 2012). Consistently, studies have suggested the regulative role of BDNF not only in the establishment but also in the formation of new synapses (Poo, 2001). The increment of axonal branches of hippocampal mossy fibres and number of dendritic spines in hippocampal pyramidal cells has been associated with BDNF signalling (Ji, Pang, Feng, and Lu, 2005).

1.2.2 Regulation of adult hippocampal neurogenesis

Adult hippocampal neurogenesis can be modulated by both extrinsic and intrinsic factors. The extrinsic factors are composed of positive regulators of AHN including environmental enrichment and voluntary exercise (Olah et al. 2009; Aimone et al. 2014), while aging and stress are related with the decrease and the inhibition of neurogenesis (Marr, Thomas, and Peterson, 2010). Diverse responses to the exposition of an extrinsic factor is caused by individual variabilities (Freund et al. 2013), which hinder the experimental assessment of the effects of extrinsic factors to neurogenesis. It has been demonstrated that proliferation of NSCs is enhanced by voluntary exercises, mainly through modification in the vasculature and the blood-brain barrier permeability (Van Praag, Kempermann, and Gage, 1999), although aging dramatically decreases the proliferation of new-born neurons in the SGZ (Eriksson et al., 1998). However, the survival of the new neurons can be promoted through environmental enrichment (Aimone et al. 2014).

Adult neurogenesis is remarkably modulated through intrinsic factors such as GABA, glutamate and the neurotransmitter systems, through activity dependent mechanisms. AHN is substantially regulated by acetylcholine (ACh) as studies have demonstrated that neurogenesis is abnormally modified when levels of ACh both increase or decrease

(Cooper-Kuhn, Winkler, and Kuhn 2004; Kaneko, Okano, and Sawamoto 2006). However, an accurate regulation of cholinergic systems has been reported, through nicotinic receptors, in the survival of new-born neurons (Berg, Neff III, Lozada, Fernandes, and Gomez-Varela, 2009). Furthermore, norepinephrine (NE) and the dopamine (DA) system modulate neurogenesis, and both neurotransmitters are required for a normal development of new-born neurons (Harley 2007; Lee et al. 2009). Dopamine and specially its receptor D2 have been related to integration and maturation of immature neurons in the hippocampus (Mu, Zhao, and Gage, 2011). A prominent regulative role has been also reported for serotonin (5-HT), which has been deeply studied due to its role in depression and stress (Warner-Schmidt and Duman, 2006). Strikingly, administration of serotonin reuptake inhibitor (SSRI), which provoke the increment of serotonin in the synaptic cleft, has been correlated with significant increase of neural progenitor's proliferation and enhancement of dendritic development (Malberg et al. 2000; Sahay and Hen 2007). Therefore, neurotransmitter serotonin is indispensable as the major modulator of the AHN.

1.3 Serotonergic system

Monoamine serotonin, or 5-hydroxytryptamine (5-HT), is found in the brain and gastrointestinal tract (Twarog and Page 1953). In the brain, 5-HT is synthetized by neurons of the brainstem, mainly located in the raphe nuclei and the reticular formation (Dahlström and Fuxe, 1964), whereas in the peripheral organs, 5-HT synthesis occurs in the gastrointestinal tract and it cannot cross the blood-brain barrier (Lesurtel et al. 2008). The location of 5-HT neurons has been classified into 9 groups, B1-B9 group cells, which are divided into two divisions: caudal (B1-B3) and rostral division (B6-B9), depending on the area of its axonal projections (Wallace and Lauder 1983) (**Figure 2A**). The rate-limiting enzyme for 5-HT synthesis is tryptophan hydroxylase (TPH) (Joh et al. 1975), which has two isoforms: TPH1 and TPH2. The neuron-specific enzyme TPH2 is confined in the raphe nuclei, while TPH1 is mostly in the peripheral organs. (**Figure 2B**) (Grenett et al. 1987; Walther and Bader 2003). Amino acid tryptophan is hydroxylated in the 5th position of the ring, followed by a decarboxylation which produces 5-hydroxytryptophan (Walther et al., 2003). After its synthesis, 5-HT is transported into synaptic vesicles through the vesicular monoamine transporter 2 (VMAT 2). Serotonin can be reuptake into the presynaptic cell by the serotonin transporter (SERT), hence facilitating the

extracellular regulation of 5-HT (Charnay and Léger, 2010). Additionally, serotonin function is mediated through a rich diversity of receptors, which consists of 15 different genes encoding 5-HT receptors classified into seven families (Gaspar, Cases, and Maroteaux, 2003).

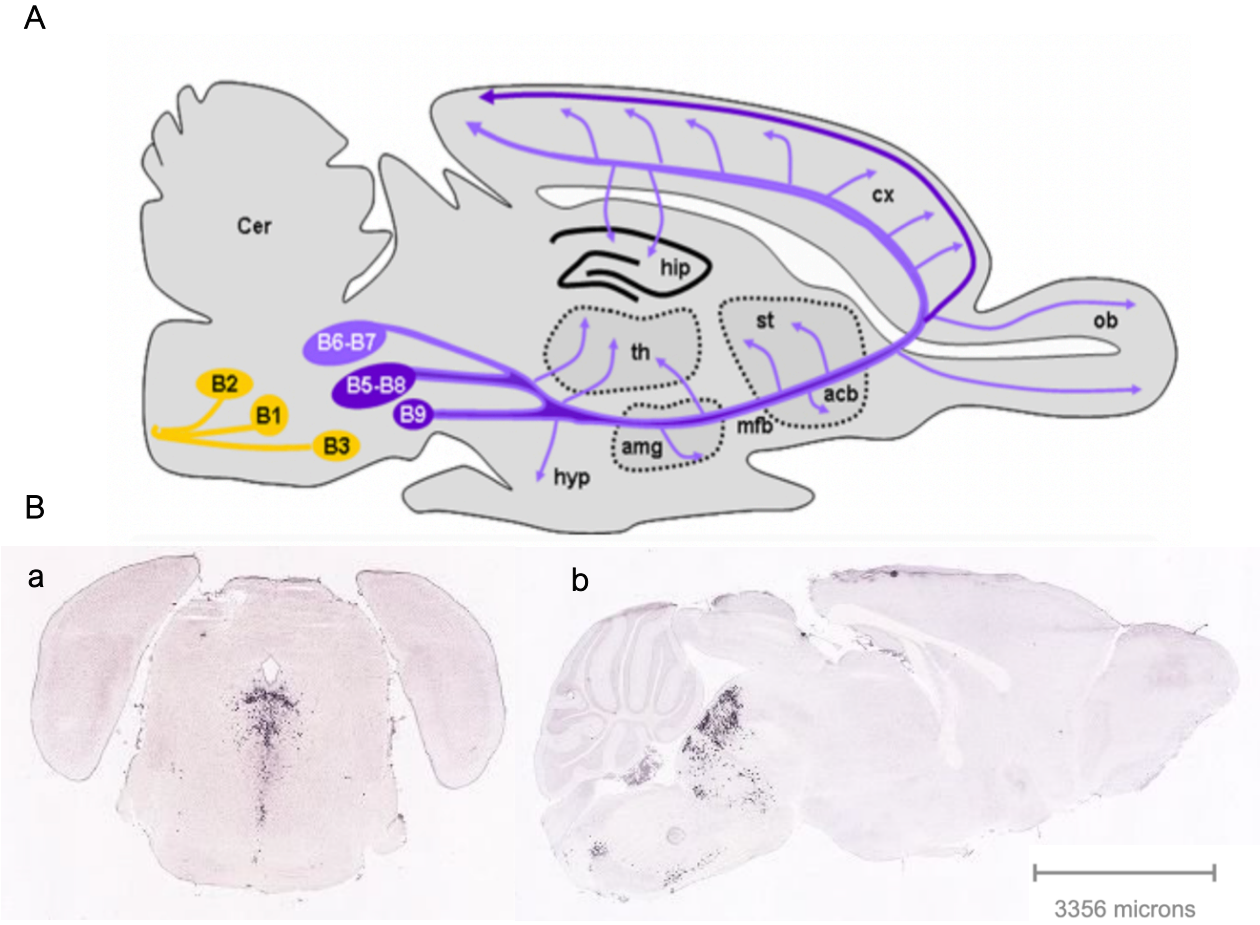


Figure 2 | Schematic illustration of serotonergic neurons' organization in the mouse brain and *Tph2* mRNA location. A | In the sagittal representation of an E11 brain, the nine cell groups (B1-B9) are indicated. In yellow are shown B1-B3 caudal groups of serotonergic cells, which project to the medulla, the spinal cord, and the periphery system. The rostral B6-B9 groups of cells (purple) send their serotonergic innervation to the telencephalon and diencephalon. Cer: Cerebellum; hyp: hypothalamus; th: thalamus; amg: amygdala; mfb: median forebrain bundle; st: striatum; acb: nucleus accumbens; ob: olfactory bulb; cx: cerebral cortex. Adapted from (Vitalis, Ansorge, and Dayer, 2013). **B** | In situ hybridization of *Tph2* mRNA of 56 weeks old C57BL/6J mice. In the coronal section **(a)** only the rostral group of cells can be observed. Both groups of serotonergic cells, rostral and caudal, can be observed in the sagittal section **(b)**. Scale bar is 3356 µm. Figure adapted from (Allen Mouse Brain Atlas 2019).

Serotonin serves an extensive neuromodulatory function in brain development. This hypothesis has been based on the early synthesis of serotonin and its extensive innervation

towards several brain areas through massively diffused collateralization of their axons (Turlejski 1996; Gaspar, Cases, and Maroteaux 2003). Serotonin not only enhances its synthesis and increases axonal development (De Vitry et al. 1986; Galter and Unsicker 2000b), but it also inhibits the differentiation of other neural precursors into serotonergic neurons, demonstrating an autocrine and paracrine effect of 5-HT (Branchereau, Chapron, and Meyrand 2002). It has been reported that 5-HT plays a major role in synaptogenesis, neurite outgrowth, and cell survival and can work as a trophic factor (Gaspar et al., 2003). In addition to the regulatory role of 5-HT in brain development, serotonin and its multiple interactions with other neurotransmitters have been related to vital physiological functions in mammals, such as reward, sleep-wake cycle, memory and cognition amongst others (Gould 1999; Charnay and Léger 2010). The neuroplastic functions of 5-HT, which is crucial in AHN, and its predominant role in depression and other pathophysiology processes have caught the major attention (Duman and Aghajanian, 2012).

1.3.1 Serotonin and adult hippocampal neurogenesis

The modulatory effect of 5-HT in the AHN has been clearly described (Malberg et al. 2000; Santarelli et al. 2003), but the mechanisms whereby serotonin regulates neurogenesis and its divergent role throughout the neural development have been reasons for discordance (Banasr et al. 2004; Ohira and Miyakawa 2011). Serotonin, through its receptors, modulates the dendritic spine density of newborn neurons, which is considered crucial for the maturation of GCs and the homeostasis of adult neurogenesis (Yan, Wilson, and Haring, 1997). Enhancement of neuronal proliferation in the adult SGZ has been reported after chronic administration of SSRI such as fluoxetine, which directly increases levels of serotonin and acts through 5-HT_{1A} receptor, the predominant mediator of serotonin action (Banasr et al. 2004; Radley and Jacobs 2002). Subsequent studies regarding the function of serotonergic projections to the hippocampus elucidated direct and indirect substantial effects of 5-HT on AHN. Projected from the dorsal raphe nucleus, serotonergic neurons directly contact with the neurogenic niche in the SGZ, diffusing 5-HT and promoting the proliferation (Kosofsky and Molliver, 1987). Concurrently, hippocampal interneurons in the DG receive inputs from the serotonergic axon terminals of the median raphe nucleus, which indirectly regulate the maturation of the new-born neurons (**Figure 2A**) (Freund et al. 1990). Moreover, it has been shown that serotonin

receptors are differentially expressed in GCs throughout their differentiation and maturation, as well as in the hippocampal interneurons. Hence, serotonin may play a homeostatic role in AHN through the differential expression of its receptors (Klempin 2010; Diaz et al. 2013; Alenina and Klempin 2015).

1.3.2 Serotonin and BDNF

Serotonergic system and BDNF-TrkB signalling system co-regulate the synaptic plasticity, neurogenesis, and correspondingly co-regulate one another (Mattson, Maudsley, and Martin, 2004). Neurogenesis has served as a convergent point for both signalling systems; while 5-HT is a primordial requisite for the AHN (Brezun and Daszuta 1999; Djavadian 2004), BDNF-TrkB signaling is essential for their survival and to ensure the functional integration of the newborn neurons into the circuitry (Sairanen, 2005). 5-HT triggers the expression of BDNF, and BDNF promotes the development and survival of serotonergic neurons (Mattson et al., 2004). BDNF induces the expression of serotonergic markers in 5-HT neurons in the raphe nucleus and governs the differentiation and survival of serotonergic neurons (Martinowich and Lu, 2008). Additionally, when *Bdnf* gene is deleted in late developmental stages through conditional knockout mice, severe deficits in the 5-HT receptor functions have been reported (Rios et al., 2006).

Molecular studies have suggested that the production of the second messenger cAMP drives the molecular co-regulation between 5-HT and BDNF. The production of cAMP, due of TrkB activation in 5-HT neurons, triggers the activation of protein kinase A (PKA), which activates the transcription factor CREB, enhancing BDNF synthesis (Galter and Unsicker, 2000a). Concurrently, studies suggest that epigenetic modifications may play a substantial role in the co-regulatory feedback loop of BDNF and 5-HT (Judith Regina Homberg, Molteni, Calabrese, and Riva, 2014). Indeed, the increase of DNA methylation and the reduction of H3 acetylation of *BDNF* promoter in 5-HT transporter knockout rats has been reported (Molteni et al., 2010), while *in vitro* studies have reported that *Bdnf* gene expression is enhanced through histone deacetylase inhibitors (Morita, Gotohda, Arimochi, Lee, and Her, 2009). Although specific serotonergic receptors such as 5-HT_{1A} may mediate the epigenetic regulation of *Bdnf* gene and, therefore, be crucial in the co-regulation between BDNF-serotonin, the relevance of the epigenetic modification and the role of 5-HT receptors remain unknown (Maya Vetencourt et al. 2011; Homberg et al.

2014). The BDNF-5-HT duo and their substantial role in neural plasticity have been deeply studied in rodent using the environmental enrichment paradigm.

1.4 Environmental enrichment

An enriched environment (EE) provides inanimate and social stimulation, that consists of spacious cages where a numerous group of animals interacts with diverse objects, such as nesting material, plastic shelters and running wheels (Mark R. Rosenzweig et al. 1978; Sztainberg and Chen 2010) **(Figure 3**). Compared to standard housing conditions, EE increases motor, cognitive, and sensory stimulation through social and physical interaction with the environment (Nithianantharajah and Hannan, 2006). Upon the establishment of the EE paradigm several neuroplastic and protective effects have been reported (Rosenzweig 1966; Rosenzweig and Bennett 1969). Animals in EE have shown an increase in neural plasticity (van Praag, Kempermann, and Gage, 2000), better performance in learning and memory tasks (Sale, Berardi, and Maffei, 2009), and enhancement of AHN (Kempermann, Kuhn, and Gage 1997; van Praag, Kempermann, and Gage 2000).

Figure 3|Picture of the EE cage used in our study. A side view of the EE cage with the metallic running wells, plastic pipes, and shelter toys.

Over the last years, the multiple benefits caused by EE have been described through a diverse number of behavioural tests. EE has been proven to provoke improvement in memory performances both spatial and non-spatial (Bruel-Jungerman, Laroche, and

Rampon 2005; Huang et al. 2006), which can be assessed in rodents through behavioural tests, such as Morris water maze test (Morris, 1981), and novel object recognition test (Mesa-Gresa, Pérez-Martinez, and Redolat, 2013). Studies have shown that robust anxiolytic-like effects are induced already after 3 weeks of EE (Roy et al. 2001; Benaroya-Milshtein et al. 2004; Leger et al. 2015), and to assess these effects, open field test or light-dark test are widely used (Van de Weerd et al. 2002; Hascoët and Bourin 2009). Likewise, antidepressant-like effects have been reported after EE exposure (Llorens-Martín et al. 2007; Hattori et al. 2007), using forced swim test or tail suspension test (Bogdanova et al. 2013; Leger et al. 2015).

Although the benefits of EE in rodents have been well-defined, the underlying molecular mechanisms whereby EE modulates its effects are still not fully understood. Serotonin has been found as indispensable to induce the benefits of EE caused by physical exercise, running wheel activity (Klempin et al. 2013; Song et al. 2017). Additionally, several studies have been focused on the putative role of AHN in the inducement of EE effects; EE not only promotes the proliferation of neural progenitors in SGZ but also increases the spine density and dendritic arborization in CA1 and DG (Rampon et al. 2000; Leggio et al. 2005; Hüttenrauch, Salinas, and Wirths 2016). Interestingly, the generation of new neurons in the hippocampus is not required to induce the effects of EE, suggesting multiple pathways and neurogenesis-independent mechanism (Meshi et al., 2006). However, the upregulation of *Bdnf* has been defined as the convergent point of different underlying molecular mechanisms of EE (Gómez-Pinilla et al. 2002; Adlard, Perreau, and Cotman 2005; Kuzumaki et al. 2011). In addition to *Bdnf*, other genes have been found as putative effectors of EE. Whereas genes belonging to chaperone families related to protein processing, and synaptic plasticity are upregulated after EE (Farmer et al. 2004; Hu et al. 2009; Nishijima, Kawakami, and Kita 2013), the expression of genes encoding microglial activation and neuron loss such as prostaglandin D2 has found decreased (Hüttenrauch, Salinas, and Wirths 2016). The identification of novel genes underlying the EE effects using transcriptome studies could help to depict the molecular mechanisms underlying EE.

Overall, serotonin and BDNF-TrkB signalling seem to be the convergent point of AHN and in the effects of EE. When BDNF-TrkB signalling is disrupted, as in conditional knockouts mice lacking either *TrkB* or *BDNF* gene, EE-induced neurogenesis is impaired,

suggesting the primordial role of BDNF-TrkB in neurogenesis and the sensitivity to antidepressant treatment (Rossi et al. 2006; Li et al. 2008).

1.5 Conditional knockout animals

Gene knockout is a commonly used technique to define the function of a specific gene *in vivo* by studying the loss of its function. By genetic engineering, the specific locus of the target gene is modified. However, the deletion of the gene in early developmental stages may lead to severe malformation or lethality. Homozygous knockout mice for *TrkB* receptor and *BDNF* produce deformities and premature death soon after birth suggesting clear evidence of the primordial role of TrkB-BDNF signalling in neural development (Klein et al. 1993; Erickson et al. 1996; Hall, Limaye, and Kulkarni 2009). Therefore, it has been essential for the development of new gene knockout techniques and the increment of their versatility. Through the establishment of heterozygous animals or the creation of inducible tissue-dependent knockout mice lines, the role of BDNF-TrkB signalling has been allowed to study (Lindholm and Castrén 2014).

2. HYPOTHESIS AND OBJECTIVES

BDNF and its receptor TrkB play a primordial role, amongst other developmental processes, in the generation of new-born neurons in the hippocampus. Concurrently, serotonin has also been defined as a key regulatory factor of AHN. Together, BDNF-TrkB and serotonin not only regulate each other but also govern underlying molecular mechanisms of AHN and may be related to antidepressant-induced mechanisms. EE has been reported to induce AHN, hence it has been widely used as a paradigm for this study. Unpublished results from the conditional knockouts mice used in this study, in which BDNF-TrkB signalling is compromised from the serotonergic neurons, indicated the impairment of AHN.

Our hypothesis was to study if EE restores the behavioural deficits and the abnormal regulation of AHN in our conditional knockout mice.

To test that hypothesis, the specific objectives were:

1. To evaluate the efficacy of EE by a behavioural battery of tests and assess the learning and memory, anti-depressive, and anxiolytic-like effects of EE.

2. To investigate the effects of EE on AHN and the putative underlying epigenetic modifications by cellular and molecular methods.

3. MATERIALS AND METHODS

3.1 Experimental animal model

The Tph2CreERT2 mice line was kindly provided by Professor Pierre Chambon´s laboratory, France. The TrkBflox mice in the C57BL/6 background were obtained from Jackson Laboratory, USA. The mice with C57BL/6J background were bred and maintained in the animal facilities of the Laboratory Animal Center (LAC) of the University of Helsinki, Finland. The double transgenic mice line was generated by cross-breeding the two strains; the tamoxifen-dependent Cre recombinase under Tph2 promotor expression line together with the mice strain containing *TrkB* flanked by loxP sites. These mice were inbred further with multiple crossings to achieve the genotypes, such as Tph2CreERT2:TrkBflox and TrkBflox (**Figure 4A)**. For all the experiments, the control and experimental group used were littermates.

Tamoxifen-dependent Cre recombinase, $CreER^{T2}$, is widely used for the establishment of conditional knockout mice lines (Feil, Valtcheva, and Feil, 2009). In this method, the ligand-binding domain of the estrogen receptor (ER^{T2}) is modified to bind tamoxifen, which is fused to Cre recombinase and produces a cytoplasmic protein, $CreER^{T2}$ (**Figure 4B**). The expression of $CreER^{T2}$ in our mice is under the promotor of Tph2 (**Figure 2B**). The resulting protein is located in the cytosol, where heat shock protein 90 (Hsp90) promotes its degradation. However, tamoxifen, an estrogen receptor antagonist, forces the dissociation of Hsp90 and ER^{T2}, allowing the translocation of $CreER^{T2}$ into the nucleus. Once in the nucleus, $CreER^{T2}$ recognize a 34-base-pair site called loxP and catalyses reciprocal conservative DNA recombination between same-oriented pairs of loxP sites (Madisen et al., 2010). The recombination leads to the elimination of the sequence flanked by two loxP sites, the target gene; *TrkB* in our study (**Figure 4B**).

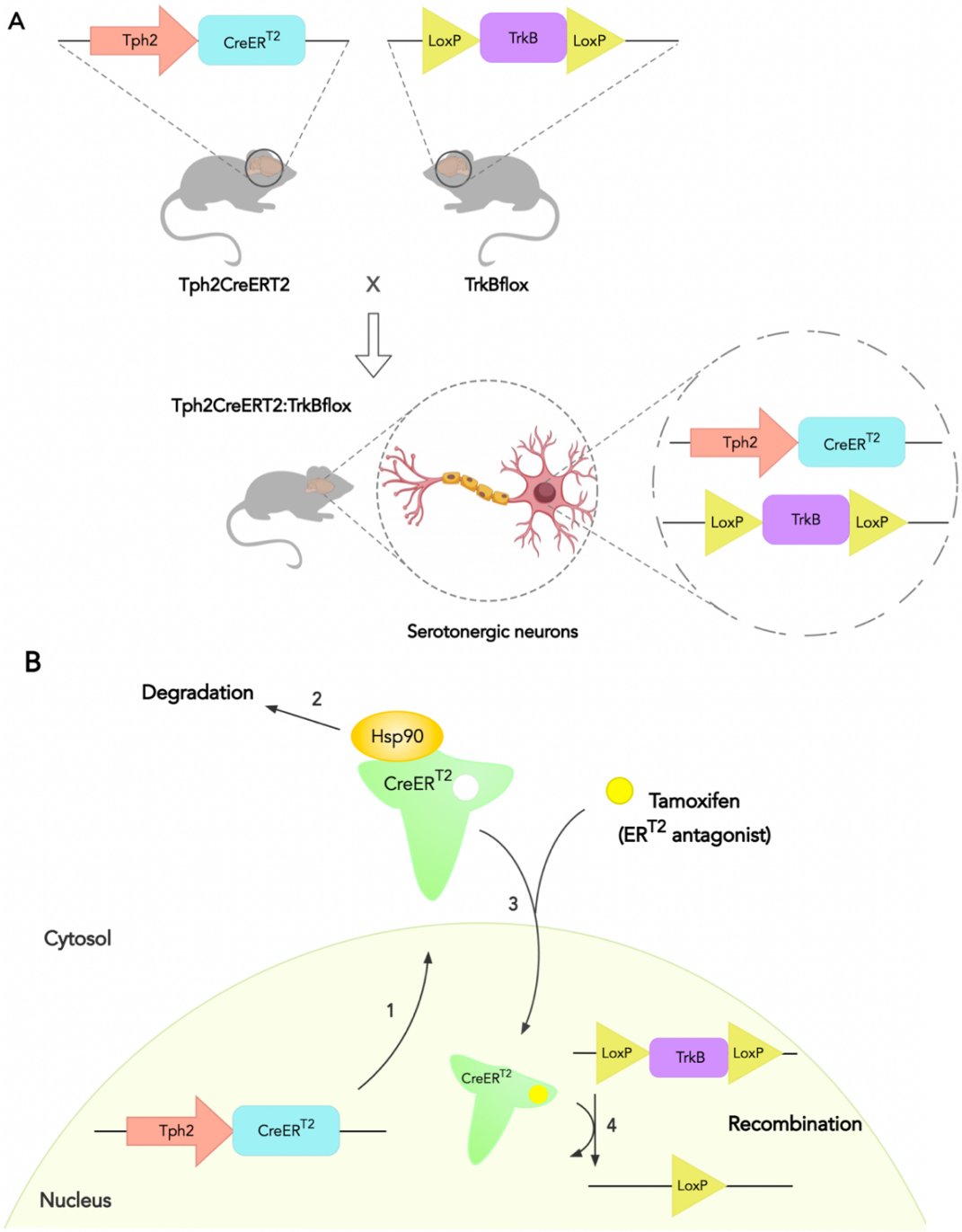


Figure 4 | Inducible tissue-specific knockout mouse model using tamoxifen-regulated gene expression. **A** | Generation of the double transgenic mice line Tph2CreERT2:TrkBflox by crossbreeding between the tamoxifen-dependent Cre recombinase under *Tph2* promotor expression line and the mice strain containing *TrkB* flanked by LoxP. **B** | Exclusively in serotonergic neurons, *Tph2* promoter triggers CreER^{T2}, which is confined in the cytosol after synthesised **(1)**. Degradation **(2)** is promoted by Hsp90, which interacts with CreER^{T2} in the cytosol. Tamoxifen irrupts the interaction between Hsp90 and CreER^{T2}, allowing its translocation to the nucleus **(3)**. The recombination **(4)** and subsequent deletion of *TrkB* occur when CreER^{T2} recognises LoxP sites.

The double transgenic mice line was administered with tamoxifen at week 6 after birth in order to ensure a normal critical period development of the nervous system. Previous studies in our lab using reporter mice have suggested the deletion of *TrkB* gene in serotonergic neurons upon tamoxifen treatment is achieved after one month. The impairment of BDNF-TrkB signalling in the serotonergic system leads to hyperactivity and disrupted feeding behaviour. Furthermore, these mice present abnormal neurogenesis, which provokes an aberrant proliferation of progenitor cells.

3.1.1 Animal license

All performed procedures in this project were in agreement with Government Decree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013) and European Union Directive 2010/63/EU. The National Animal Experiment Board (ELLA) approved the project, and all animal procedures were conducted following the guidelines from the ethical committee of Southern Finland with license number- ESAVI/10300/04.10.07/2016.

3.1.2 Cre activation using tamoxifen

To activate Cre recombinase six-week-old mice were injected with tamoxifen. The solution was prepared by dissolving tamoxifen (Sigma-Aldrich, **table 1**) in corn oil (Sigma-Aldrich, **table 1**) at a concentration of 20 mg/ml, by shaking overnight at 37 ºC. Due to the light sensitivity of tamoxifen, opaque tubes were used for preparing the solution, and to ensure the complete suspension of tamoxifen, corn oil was previously heated to 60 ºC. To preserve the quality, the ready solution was stored at 4 ºC and used within three weeks. The mice were weighed before performing the injection. Any mice that weighed under 12 g were excluded from tamoxifen injections. A standard dose of 100 µL tamoxifen solution was injected every 24 hours for four consecutive days. Due to the toxicity of tamoxifen, the intraperitoneal injections were performed in a biological safety cabinet class II using appropriate protective gears. Moreover, water and food were unchanged for one week to reduce contamination in the animal facility. Although no pain was expected, animals were monitored for two weeks. Previous studies conducting tamoxifen injections showed mice survive without any side effects on their feeding or social behaviours.

The Tph2CreERT2:TrkBflox mice were intraperitoneally injected with tamoxifen to activate the Cre-dependent recombination, which leads to the complete establishment of the conditional knockout. The Tph2creERT2 line was hemizygous while TrkBflox mice were maintained as homozygous. The littermates consisted of two genotypes, such as Tph2CreERT2:TrkBflox and TrkBflox. The control group, TrkBflox mice, were also injected with tamoxifen.

3.2 Animal housing conditions

The Tph2CreERT2:TrkBflox 9-10 months-old male and female mice were used in all the experiments. For 10 weeks, food and water were provided *ad libitum*, in a room with reversed 12 h light-dark cycle (18:00 - 6:00), regulated temperature (21 ± 1 ºC) and humidity (55 ± 10%). To validate the results, control and transgenic mice of both genders were caged in two types of housing conditions: standard housing (SH) and enriched environment (EE).

3.2.1 Standard housing condition

Under standard housing conditions, control (n = 32) and transgenic (n = 28) mice were caged in standard individually-ventilated cages (IVC) (37 cm x 19 cm x 13 cm) for 10 weeks in group of 2-3, segregated by sex. Neither toys nor running wheels were included in standard housing condition, only social interaction was allowed. They were maintained in the same room with the EE mice in Scantainers.

3.2.2 Enriched environment housing condition

Larger polycarbonate cages (90 cm x 90 cm x 32 cm) were used for caging control (n = 11) and transgenic (n = 13) mice under EE-living conditions. Although it has been previously proved that long-term enriched housing leads the increment of aggressive behaviour of male mice which leads the exclusion of male in EE experiments (Marashi, Barnekow, Ossendorf, and Sachser, 2003), we decided to use both sexes to increase the external validity of our study. Therefore, females and males were maintained in two EE cages segregated by sex. Running wheels, tunnels, plastic toys, small gnawing wooden bricks and extra nesting material were included in EE cages. These were cleaned and rearranged, once in three weeks, to increase the sense of novelty. However, a small

amount of old bedding and nesting material were maintained in males cage to maintain the organizational hierarchy. If any aggressive behaviours were reported among the male groups during our experiment, the susceptible subjects were transferred to individual rat cages, equipped with a running wheel, a small tunnel, and a plastic shelter.

3.3 Behavioural battery

To assess the putative effects of EE, we conducted a battery of behavioural tests with the animals. The behavioural experiments included in the battery were: light-dark box, forced swimming, marble burying, novel object recognition, pre-pulse inhibition of the startle response, and cued and contextual fear conditioning. After six weeks of EE, the first experiment of the behavioural battery test was conducted. Throughout the following four weeks, the behavioural tests were performed following a hierarchical strategy for behavioural characterization in which experiments were conducted with the least stressful to more stressful in order to reduce the interaction between experiments. All the behavioural tests were done in similar test rooms conditions: soundproof rooms, extreme silence, and the light was ~ 150 lux, except for novel object recognition test (~ 20 lux). Before the beginning of the behavioural experiments, mice were transferred to the experimental room for 30 minutes for habituation. We have used both sexes in the experiments and to avoid interference between trials, experiments were firstly conducted with males followed by females. Furthermore, all the behavioural tests were performed with researcher blinded to the genotypes of the animal to avoid bias.

3.3.1 Light-dark box test

The light-dark box (LD) test is based on two rodent behavioural characteristics; the inherent preference for dark limited spaces compared to brilliantly open areas, and the natural exploratory behaviour (Crawley and Goodwin, 1980). A conflict between the innate preference and the spontaneous exploratory behaviour is created when mice are exposed to a novel but illuminated open arena. Thus, the avoidance of its exploratory behaviour under mild stressor conditions, as illuminated areas, should indicate the level of anxiousness. Consequently, LD became an easy, quick and reliable model to assess anxiolytic-like behaviours in rodents (Hascoët and Bourin, 2009).

A brightly illuminated (~ 550 lux) polypropylene chamber (30 cm x 30 cm x 30 cm) was used as test arena. In order to establish the dark and light compartment, an opaque box insert (15 cm x 15 cm x 30 cm) was introduced to the open arena. The division of the two compartments was unequal: the bright arena occupied two thirds, and the dark side was the remaining one third. The compartments were connected through a small opening (7 cm height x 5.5 cm width) located on the black insert wall, so the animals could move freely between compartments. The chamber was equipped with three pairs of infrared strips and sensors, located on the sides of the chamber, so three axes (X, Y, and Z) were tracked during the trials, and all horizontal and vertical movements were scored. Infrared beams passed through the dark box insert, although it was completely opaque to light. The infrared strips and sensors of different eight arenas were simultaneously connected to the Activity Monitor System (Med Associates Inc, **table 1**), which monitors several different movement features throughout the trials.

The animals were gently placed into the dark compartment through the opening situated on the top wall of the dark box insert and, immediately, the Activity Monitor System started to track the movements along the compartments. During the trials, neither noise nor light variation were allowed. After 10 minutes had elapsed, the Activity Monitor System automatically stopped tracking, and the mice were kindly removed from the arena to their home cages. Faeces were removed after the trials, and the arenas and dark-box inserts were cleaned with ethanol (70% vol/vol) and water using paper towels.

The Activity Monitor Software was used to analyse and export the data for further analysis. The software assessed up to 14 different parameters, amongst which there were vertical counts, jump counts, and ambulatory distance, referred also as distance travelled. Defined as the Euclidean approximation distance of all the ambulatory count measured in beams, the distance travelled was the main parameter used to assess the exploratory behaviour of mice.

3.3.2 Marble burying test

The marble burying (MB) test is based on the innate digging and burrowing behaviours of rodents (Deacon, 2006a), including non-aversive objects such as glass marble (Broekkamp, Rijk, Joly-Gelouin, and Lloyd, 1986). It has been demonstrated that compulsive or anxiolytic-like behaviours influence burying spontaneous behaviour.

Whereas decreasing of burying behaviour has been associated with anxiolytic-like effects after antidepressant treatments, defensive burying behaviours were related to stress-induced paradigms and aversive stimuli (Poling, Cleary, and Monaghan 1981; Broekkamp et al. 1986). Thus, MB has shown to examine anxiety or compulsive burying-like behaviour in rodents. As previous findings indicated no behavioural difference in our transgenic mice, no change was expected in this paradigm.

The experiment was conducted using IVC500 clean cages without food racks or its corresponding lids. The cages were filled with 5 cm of chipped aspen bedding material, creating a flat thick surface in the cage. Special measures were taken to avoid differences within the thickness of the bedding material layer between test arenas. Twenty glass marbles (15 mm diameter) were carefully distributed on the surface of the bedding, in an evenly 4 x 5 disposition. Since the standard cage lid was unsuitable to use due to the thickness of the bedding material, a rectangular polypropylene surface was used to cover the cage. No video tracking software was used in the test and the measurements were done manually.

First, the animals were habituated in the cages with the bedding material for 10 minutes, allowing them to explore the cages. After habituation, mice were gently placed back to the home cages. The bedding was smoothened, and the glass marbles were placed in the arena as previously mentioned. The animals were then returned to the respective cages, which were carefully closed, and the testing phase took place for 15 minutes. Four trials were simultaneously conducted, and the cages were maintained so no interaction between cages was possible. The bedding material was completely renewed between trials, and cages were cleaned with ethanol (70% vol/vol), water and paper towels. Previous studies in our lab suggested that measurements should be done in intervals of five minutes, rather than a single final measurement after 15 minutes. Thus, the number of buried marbles were counted every five minutes, classifying the marble into three categories: completely buried - when marble was no longer visible; half-buried - at most half glass marble was visible without removing the bedding material, and unburied - when the marble was on the surface and slightly covered or uncovered by bedding.

3.3.3 Novel object recognition test

Novel object recognition (NOR) test is based on the rodent innate preference for novel objects, in a certain environment, and its associated explorative behaviour (Berlyne 1950; Ennaceur 2010). The versatile test assesses different aspects of memory and learning such as acquisition, consolidation, short- or long-term memory and other different types of memory (Lueptow, 2017). Furthermore, the test is widely used to measure the effect on learning and memory caused by drugs, hormones or EE (Goulart et al. 2010; Cyrenne and Brown 2011; Viola et al., 2010; Melani et al., 2017).

The test was conducted in two parallel square open field (30 cm x 30 cm x 20 cm), which included a plastic grey floor and white opaque walls. White table-tennis balls (~ 12 cm^3) and black oval balls (~ 12 cm^3), similar in shape but contrasting in colour, were used as experimental objects. Differences between the two types of objects were perceptible by mice, although the shape and texture were similar in order to avoid induced object preference (Ennaceur, 2010). Marks on the floor were made to identify the exact position of the objects, so variation between trials was reduced. The experiment was performed under reduced light to lower the stress of the animals during trials, and to improve EthoVision XT13 tracking outcomes. The arenas were indirectly illuminated using four dimming light bulbs on the walls, which shined light on the ceiling. The camera connected to the Video-Tracking System EthoVision XT13 recorded the trials and was placed directly overhead the arenas. Before starting the test, the background settings were modified to facilitate the detection of mice during trials. Likewise, the EthoVision XT13 arena settings were modified in such a way that the object exploration zone was set within a 10 cm diameter around the object, and all the arenas and objects were carefully labelled for further analysis.

The test was conducted in three phases: habituation, familiarization, and test session. In habituation, mice were gently placed in the centre of the open field, without any object, and were allowed to freely explore and habituate to the arena for five minutes. Since our aim was to assess the memory and learning improvements, the habituation phase was not recorded. After five minutes has elapsed, mice were returned to their home cages, and the apparatus was thoroughly cleaned with ethanol 70% (vol/vol) and water.

During familiarization session, mice were let to explore and interact with two similar objects. Although this phase is commonly performed 24 hours after the habituation session (Leger et al., 2013), we decided to perform it after 2 hours as previous results from our group have suggested more reliable results. Additionally, it has been demonstrated that rodents spend more time exploring the novel object during the first few minutes of the phase (Antunes, M. and Biala, 2012), thus the length of familiarization and test session were reduced from ten to six minutes to avoid preference bias. Starting with the familiarization phase, two identical white table-tennis balls were diagonally placed in each arena, keeping ~ 5 cm from the walls. Subsequently, mice were gently placed in one corner of the arena, facing the wall, and allowed to interact with the objects for six minutes. All the movements were tracked using both nose-point and centre-point detection of EthoVision XT13. After six minutes, the Video-Tracking System automatically stopped the recording and mice were carefully removed back to their home cages. Special efforts were made when cleaning the objects to avoid any kind of olfactory clues between trials. Therefore, two batches of different objects were used. Moreover, the faecal material was removed, and the arenas were carefully cleaned. When handling the animals, extra care was taken to avoid any stressful situation, which could affect the spontaneous explorative behaviour of mice (Deacon, 2006b).

Twenty-four hours later, the test session was performed, which required the same arena settings as the previous session. One familiar object - white tennis table ball, was replaced by a novel object - black oval ball-. Mice were then freely let to explore the arenas and interact with the objects for six minutes. All the test sessions were recorded by EthoVision XT13.

In familiarization and test session, the Video-Tracking software measured different parameter such as distance travelled, time spent in object exploration zone and time spent interacting with novel and familiar object. Exploration in rodents is defined as “when the mouse’s nose is pointed towards the object within 2-3 cm of the object, with active vibrissae, sweeping or sniffing”, and it is excluded from exploration “the time mice spent sitting next to the object without active exploration” (Lueptow, 2017). With EthoVision XT13 Software´s reduced detection limits, nose poke might be concealed and confused with other mice movements such as tail movements. Previous studies have shown strong discrepancies between EthoVision detection of nose-poke measurements and manually

analysed data. Thus, nose poke and interaction between mice and the objects were manually analysed by visualising the video trials. Researcher was blinded for the genotype and the group during the analysis.

3.3.4 Cued and contextual fear conditioning test

The cued and contextual fear conditioning paradigm (FC) assesses the associative learning in fear-evoking contexts. The test is based on pairing an aversive unconditioned stimulus (US), such as a foot shock, with a conditioned stimulus (CS), as a tone and a particular context (Curzon, Rustay, and Browman, 2009). The passive associative learning leads the expression of fear responses when the neutral conditioned stimulus, the tone or the context, is presented (Flor and Birbaumer, 2001). The induced fear is exhibited in rodents as freezing behaviour, which leads to static and defensive postures. Therefore, FC is widely used to measure the ability of rodents to learn and recall the association of aversive experiences and environmental cues (Shoji, Takao, Hattori, and Miyakawa, 2014). The critical role of conditioned fear in depression and anxiety disorders has been demonstrated, hence the study of fear acquisition, fear consolidation, and fear extinction became essential for the understanding of these disorders and their treatments (Judith R. Homberg, 2012). The protocol described in Karpova et al. (2011) was applied in our study.

The computer-controlled Fear Conditioning System (TSE System, **table 1**) was used for studying of cued and contextual fear responses. The system comprised two boxes, each one includes a detection sensors frame (three-dimensional infra-red-light transmitter and sensors), a removable stainless-steel grid-floor (bar ∅ 4 mm, 10 mm distance) and the test arena. The test arena varied according to the context, such as context A, a translucid Perspex (acrylic) arena with a lid, and context B, a black opaque arena with a PVC plate used as a smooth floor. The boxes were also equipped with a loudspeaker, light source, and ventilator. Underneath the shock grid, a stainless-steel removable faeces tray was placed. The boxes were connected to the control unit, which configures the sound and noise generator, the amplifier, the light source, and the shock grid. The control interface, through which the experiment was performed, was connected to a computer. The paradigm was composed of five phases: conditioning, extinction phase 1 and 2, spontaneous recovery, and fear renewal, which were performed in two contexts.

Conditioning phase required a transparent acrylic cage (23 cm x 23 cm x 35 cm) which was placed in the sensor frame, above the grid floor. The cage was constantly illuminated (~ 100 lux) and white background noise (68 dB) was generated. Mice were introduced to the arenas, and after two minutes of free exploration with white background noise, a 10 kHz tone (76 dB and pulsed 5Hz) was emitted for 30 seconds, followed by a two seconds foot shock (0.6 mA, constant current) produced by the grid-floor. The pairing of an auditory cue and the electric shock was presented five times, within a varying interval of time, which varied from 20 to 120 seconds. Inside the cages, the time of freezing, defined as the motionless behaviour longer than three seconds, was measured through the infrared light beams at a frequency of 10 Hz. Throughout the conditioning phase, the animals were exposed to an aversive electric shock (US) followed by a loud sound (CS) and, as a result, they were fear conditioned. Moreover, as the CS-US pairing was presented in a specific context, transparent bright cage and grid-floor, animals were also contextually conditioned.

Extinction phase 1 and 2 were performed 24 and 48 hours after the conditioning phase, respectively. The novel context B was introduced by using a black opaque acrylic cage (23 cm x 23 cm x 35 cm), and a black PVC smooth plate as the floor, which was placed overlie the grid-floor. Additionally, in order to increase the novelty of the context proving a novel odour, a layer of wood chips, conventional bedding material, was incorporated in the faecal tray. Context B, therefore, differed extremally from context A, used in the conditioning phase. However, not only the context but also the contents of the phase was modified, since no foot shock was presented during extinction phases. In the extinction phases, animals were carefully transferred from their homecages to the arenas, where they were allowed to freely explore the novel context while a white background noise (68 dB) was played. Afterward, a 10 kHz tone (CS) was emitted for 30 seconds and repeated 12 times, within irregular inter-intervals times which varied from 20 to 60 seconds. During the conditioning phase, mice were scored for freezing behaviour through the infra-red-light detection system.

Spontaneous recovery phase and renewal phase were performed six days after extinction phase 2. Whereas spontaneous recovery was performed using the extinction context B, the conditioning context A was used in the renewal phase. Both in spontaneous recovery

and renewal phase, no aversive stimulus, foot shock, was delivered and exclusively a 10 kHz tone (CS) was played four times, within irregular inter-intervals times which varied from 20 to 60 seconds. Additionally, as in previous phases, the freezing time was scored. Renewal phase was conducted immediately after spontaneous recovery phase, carefully cleaning the conditioning boxes and components to reduce odour clues. Meanwhile, mice were returned to their homecages and maintained in the experimental room. Once the last phase was performed, the obtained data were exported from the computer for further analysis.

Before the start of each phase, infra-red sensors, the light source, and the electric shock generator were tested, as any malfunction could affect the scoring of the freezing behaviour. As the odours and environmental clues or stimulus are crucial in FC test, extreme caution during the cleaning of the conditioning box was taken. Once the phases were finished, mice were returned to their home cages, and the arenas walls, the metal grid, and the faecal tray were conscientiously cleaned with ethanol (70% vol/vol) and water. Additionally, after the last trial, the shock-grid was carefully removed and cleaned with ethanol (70% vol/vol) and urine and faecal material were removed. When context B was performed, the bedding material used for increasing the novelty was renewed every second trial to reduce odour clue.

3.3.5 Pre-pulse inhibition of startle response test

Pre-pulse inhibition of startle response (PPI) test is based on the physiological sensorimotor gating (Braff et al., 2001). When an intense and unexpected stimulus is presented, such as a powerful auditory stimulus, it provokes an involuntary contraction of the musculature, which is known as startle reflex. PPI is defined as specific startle plasticity and it assumes the ability of a non-startling, low-intensity pre-stimulus (pre-pulse) to reduce the startle magnitude provoked by a subsequent startling stimulus (pulse) (Graham 1975; Hoffman and Ison 1980). PPI is widely used in the assessment of sensorimotor gating performance in rodents (Carter et al., 1999). Previous studies demonstrated that sensorimotor gating is impaired in a wide spectrum of neuropsychiatric disorders such as obsessive-compulsive disorder (Ahmari, Risbrough, Geyer, and Simpson, 2012), Huntington's disease (Swerdlow et al., 1995), or schizophrenia (Powell, Zhou, and Geyer, 2009). Therefore, PPI has been stablished as a reliable paradigm for the

understanding of neuropsychiatric disorders, and the investigation of antipsychotic treatments.

The apparatus (Startle Reflex System, **table 1**) comprised two acoustic startle soundproof cubicles connected to the programmable audio generator, the sound amplifier, and a computer containing the Startle Monitor Software. Inside the acoustic startle cubicle, the apparatus was equipped with an attenuator, an amplifier, and the startle stimulus platform. Mice were introduced to an acrylic transparent cylinder (∅ 4,5 cm, 8 cm length), which was fitted on the startle stimulus platform. One lateral side of the cylinder presented several holes, which allowed the transmission of the acoustic stimulus. The acoustic stimulus was emitted through the speakers located in the startle stimulus platform, which also recorded the amplitude of the startle response of mice.

To habituate the mice to the cylinder, the acoustic startle box, and the emitted background white noise (65 dB), the animals were maintained in the cylinder for five minutes, which was considered as acclimation period. After five minutes had elapsed, the testing phase started, which was composed of three blocks. Block-1 was composed of five trials, wherein each one a startle stimulus (SS) of 120-dB was emitted for 40 ms. Block-2, however, consisted of 50 trials, where the startle stimulus could be unpreceded or preceded by the acoustic pre-pulse stimulus (PPS). A 20 ms white noise bursts of 68, 72, 76, and 80 dB were played as PPS, and background noise and startle stimulus were as defined for Block-1. Throughout the 50 trials, SS and SS+PPS were emitted in a pseudorandomized manner, as each pre-pulse level (0, 68, 72, 76, and 80 dB) was produced once every five trials. When emitted, the delay between the pre-pulse and the startle stimulus was 100 ms, and the inter-stimulus intervals varied from 8 to 20 s. In the last block, Block-3, five trials were performed, each one consisting of the emission of a startle stimulus (120 dB, 40 ms) without pre-pulse stimulus. After the onset of the startle stimulus, the startle response was recorded for 65 ms. The startle response measured for Block-1 and Block-3 were excluded from the calculation of the average of the startle response. The pre-pulse inhibition was calculated as indicated in **Equation 1**:

$$PPI_X\ (\%) = \ 100 - \left(\frac{Startle\ response\ PPS_x}{Startle\ response\ SS_X}\right)\ x\ 100$$

Equation 1 | Percentage of pre-pulse inhibition. For each PPS intensity level, *(X* = 68, 72, 72, and 80 dB). Startle response PPS_X is the average of the startle response in 10 trials when the PPS was followed by SS (120 dB), while startle response SS_x refers to the average startle response during the 10 trials when SS was unpreceded of PSS.

Mice were transferred from their homecages to the restrainers, the transparent cylinder. In order to retain the mice inside, the restrainers were closed using two road lids, which were thoroughly tightened with Blue-tack to prevent the escape. When placing the restrainer on the startle stimulus platform, we ensured that the perforated side of the cylinder was directed towards the speakers. Upon the finalization of trials, animals were carefully removed from the restrainers, which were cleaned with ethanol (70% vol/vol) and water.

3.3.6 Forced swimming test

The forced swim test (FST) is based on the response to an unavoidable stressor represented as an immobile posture adopted by mice (Porsolt, Bertin, and Jalfre, 1977). The test analyses the escape-related mobility behaviour in an inescapable cylindrical tank (Can et al., 2011). Since stress has been postulated as an enhancer of depression-like behaviour, FST became a potent tool to monitor depressive-like behaviour in rodents (Yankelevitch-Yahav, Franko, Huly, and Doron, 2015). Furthermore, induced-anxiolytic-like effects can be easily assessed in rodents through FST (Petit-Demouliere, Chenu, and Bourin, 2005).

The experiment was conducted using cylindrical plexiglass tanks (24 cm height, 13 cm diameter), which were filled up with room temperature water. To prevent animals from escaping and, concurrently, from touching the bottom of the tanks, the water level was maintained at 15 cm from the bottom of the beaker. The water was discarded and renewed every four trials and faecal material was removed after each trial. Two transparent tanks were placed next to each other, separated by an opaque divider to reduce the interactions between simultaneous trials. Additionally, behind the tanks, a white wall was required as a background for the recordings. Two parallel trials were recorded using a camera from the front, which was connected to the Video-Tracking System EthoVision XT13 (Noldus

Information Technology, **table 1**). The illumination of the tanks was carefully monitored, as small variations could interfere with the tracking procedure of EthoVision XT13.

The animals were introduced into the tanks by holding them by the tail. This was followed by the automatic detection of the animal placement inside the cylindrical tank by Video-Tracking System EthoVision XT13. For six minutes, changes on the mobility were measured using the centre point of the animal as a mean point of tracking. The video was automatically stopped after six minutes, and mice were carefully removed from the tanks. During the trials, the researcher was behind an opaque screen at a reasonable distance from the arena to avoid any disturbance or noise. To prevent hypothermia, animals were gently dried up with paper towel and carefully placed into a recovery chamber at ~ 30 ºC for approximately six minutes. Then, mice were returned to their home cages. As FST is considered a traumatic experience for mice (Can et al., 2011), the test was the last behavioural experiment performed.

Although EthoVision XT13 automatically analysed different parameters, the total immobility time was manually evaluated due to its elevated complexity to be automatically analysed. Immobility has been specified as the absence of any other movements than those necessary to keep the head above the water such as kicking one leg. Moreover, drifting resulted from the *momentum* after a burst of mobility was scored as immobility (Cryan, Markou, and Lucki, 2002). The immobility time and the latency to immobility were analysed from the last four minutes of the recorded trials. The first two minutes of the trial were considered unreliable to be scored due to mice were excessively active when placed inside the tank.

3.4 Sample collection

Brain tissue was collected after 10 weeks of EE experiment and after performing the behavioural test battery. Mice were euthanized by carbon dioxide (CO_2) inhalation following the stipulated guidelines of the Laboratory Animal Center (LAC) of the University of Helsinki, Finland. This method was appropriate for our research aims. Mice were individually euthanized in their homecages, inducing a painless and fast death. Elevated concentration of CO_2 rapidly induces anaesthetic effects, which can lead to death by asphyxiation under the unconscious state.

Cages were filled with a flow rate of two litres per minute of 100% CO_2, inducing unconsciousness in 3-4 minutes. To ensure the well-being of mice, the concentration of CO_2 must be increased gradually, as a rapid increase may cause bradycardia in rodents. Mice were kept under CO_2 inhalation for one minute after unconsciousness state was observed, which is characterised by the loss of the righting reflex (IACUC, 2016). Then, the CO_2 flow was increased to 10 litres per minute to ensure the death of animals. Due to the reversibility of the anaesthetic effects of CO_2, once mice were removed from the cages, death was assured through cervical dislocation.

3.4.1 Brain dissection

Following cervical dislocation, mice were decapitated by cutting the neck, approximately 0.5 centimetres below the ears, using student surgical scissors (Fine Science Tools, **table 1**) and leaving a small cervical segment of the spinal cord. We made a vertical cut of the skin in the posterior side of the skull, which became exposed by pulling the skin toward the skull anterior side. Remaining skin, muscle and fat tissue connected to the base of the skull were removed, so the skull was completely uncovered. Afterward, one blade of the fine sharp scissors (Fine Science Tools, **table 1**) was inserted through the cervical segments of the spinal cord, leaving one blade beneath and the other above the skull. The skull was carefully cut from the occipital bone to the anterolateral frontal bone, following the junction between the frontal and parietal bone with the squamous bone (**Figure 5, dashed line**). While cutting the skull, the scissors were gently pushed outwards, in the opposite direction of the brain, to avoid damaging the tissue. After repeating this step in the other side of the skull, the fine sharp scissors were introduced through the lachrymal and a cut was done along the anterior side of the frontal bone (**Figure 5, solid line)**. The dorsal part of the skull, which is formed by frontal, lateral, interparietal, and occipital bones, was easily removed using serrated Halsted-Mosquito hemostats (Fine Science Tools, **table 1**).

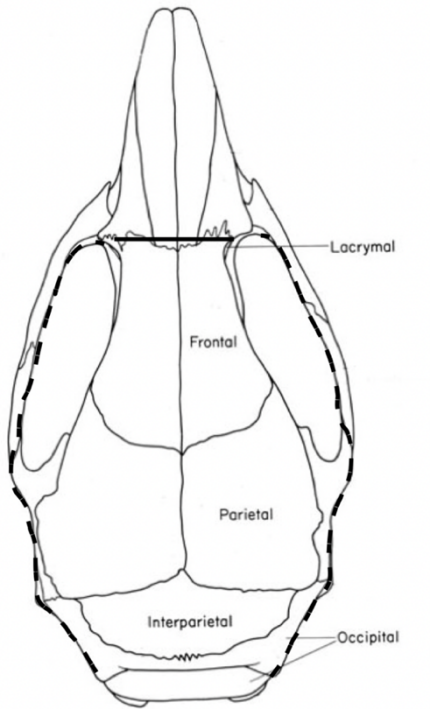


Figure 5 | Schematic representation of mouse skull's bone structure. Indicated are the principal bone plates. Dark dashed lines represent the cuts performed to remove the dorsal skull. The horizontal solid line represents the cut along the anterior side of the frontal bone. Adapted from (Spijker, 2011).

Once the brain was accessible, a small flat hippocampal dissecting spatula was carefully introduced between the skull and the lateral side of the brain. By gently tilting the brain upwards, its separation from the skull was achieved. Then, the brain was carefully pushed until the cranial nerves were broken, allowing its complete detachment. Immediately, the brain was introduced to cold RNAse-free 1X PBS to reduce the temperature and eliminate the excess of blood.

Using a spoon (Fine Science Tools, **table 1**), the brain was transferred from the cold RNAse-free 1X PBS to the surface of a filter paper moistened with sterile 1X PBS. The filter paper was located on an inverted petri dish filled with ice, which functioned as a cooling system to maintain a low temperature while dissecting. Surgical instruments were kept on ice during the dissection and carefully cleaned after each dissection with 1X PBS. Brain samples were maintained in expanded polystyrene boxes with dry ice to minimize RNA degradation during dissection. After dissection, the samples were stored at -80 ºC.

3.4.2 Prefrontal cortex dissection

The brain was placed as the ventral side facing the moistened filter paper (**Figure 6A**), helped by small curved serrated forceps (Fine Science Tools, **table 1**). Using a scalpel (Fine Science Tools, **table 1**), the olfactory bulbs were removed by cutting frontally and slightly obliquely, between the olfactory bulbs and the cerebral cortex (**Figure 6A, 1**). Subsequently, the olfactory bulbs were discarded. Two small sagittal cuts (~ 3 mm) were done in the middle of each hemisphere of the cerebral cortex (**Figure 6A, 2**). Posterior to these two cuts, a frontal cut was performed, and the tissue was gently separated from the brain using the scalpel and small forceps to hold the brain (**Figure 6A, 3**). Consequently, as indicated in **Figure 6B**, the prefrontal cortex (PFC) was easily dissected, and the tissue was collected in marked Eppendorf tubes and stored in the dry ice. In the remaining brain, a firm cut was done along the longitudinal fissure, the superior and inferior colliculus, and the cerebellum (**Figure 6C, 4**), thoroughly cutting the corpus callosum and separating the two symmetric halves.

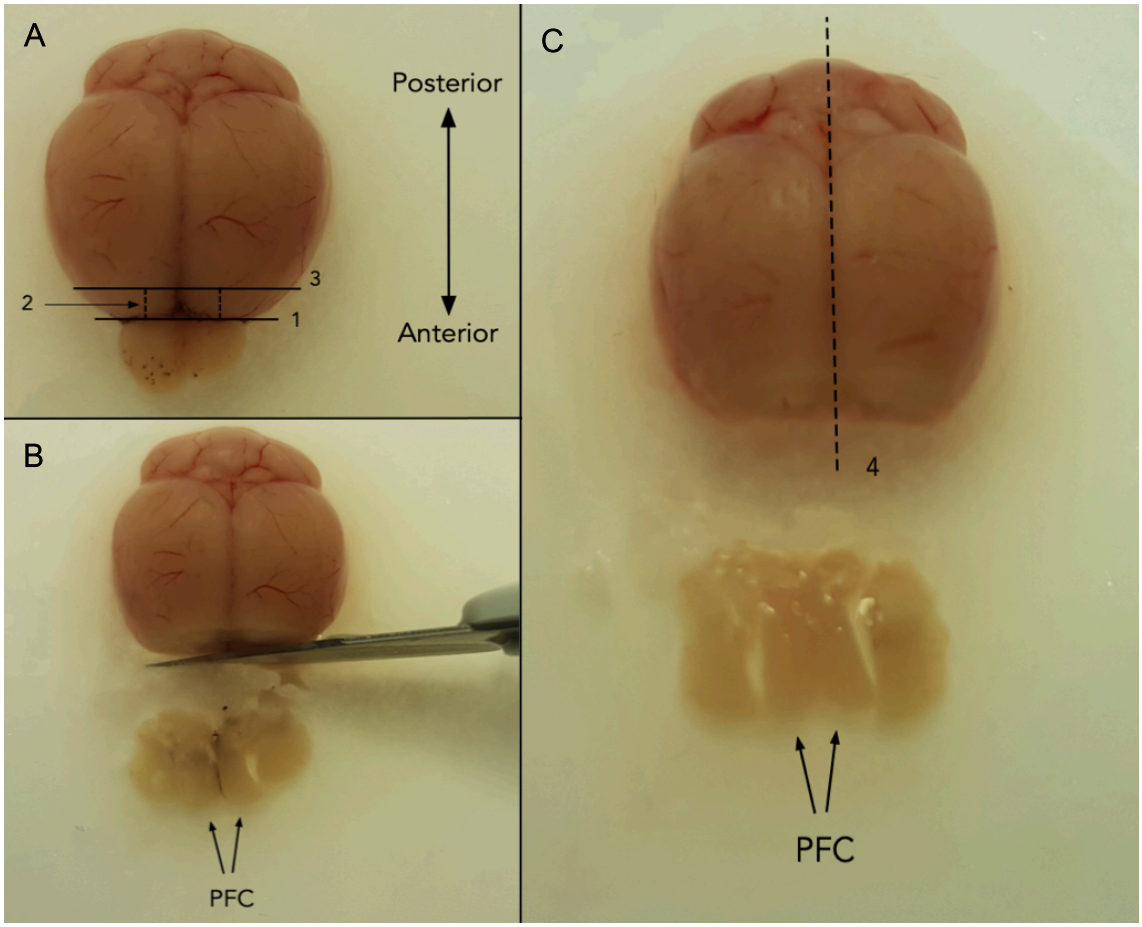


Figure 6 | Dissection of the prefrontal cortex. **A** | Lines, dashes and numbers (1, 2, and 3) indicate the location and the order of the cuts of the prefrontal cortex dissection. Additionally, the anteroposterior axis is represented. **B** | PFC is dissected and indicated

with arrows. **C**|Dashed line represents the cut along the longitudinal fissure to divide the brain into two halves to proceed with the dissection. PFC: prefrontal cortex.

3.4.3 Hippocampus and cortex dissection

Both halves were separated and placed so that the ventral side was accessible (**Figure 7A**). While the brain was held by placing the small forceps on the pons and medulla area, a brush size two was used to gently separate the cerebral cortex and the hippocampus, using the white-coloured tissue underneath the cerebral cortex as a reference (**Figure 7A, 5**). When the brush was smoothly introduced underneath the cortex to the hippocampus/cortex boundary, the hippocampus became visible as a small kidney-shaped structure (**Figure 7B**). Subsequently, to release the hippocampus, translucent and grey, it was gently pushed using the brush while the brain was carefully held with blunt forceps (**Figure 7C**).

Once the hippocampus was dissected, the ventral side of the cerebral cortex was accessible. While keeping the brain in the same position by gently placing the forceps on the anteromedial side, the brush was placed on the striatum around the perimeter of the striatum area (**Figure 7D, 6**). By smoothly pressing and rolling the brush, one-half of the cerebral cortex was removed (**Figure 7E**). Thereafter, the same steps were repeated for the other half-brain, obtaining two hippocampus and two cortex samples, which were collected in marked Eppendorf tubes and stored in the dry ice.

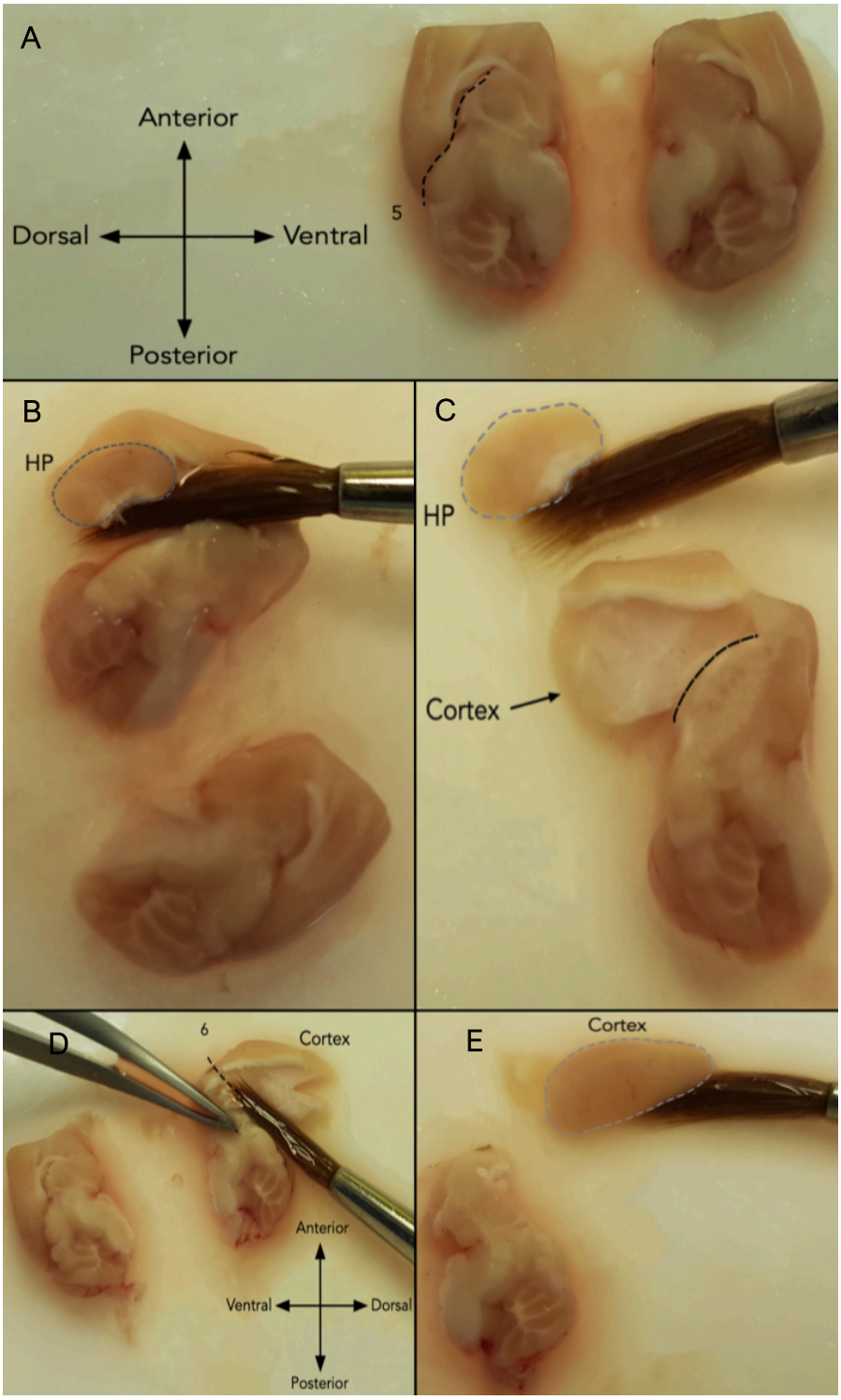


Figure 7|Dissection of the hippocampus and cortex. **A|**Dashed line indicates the location where the brush was introduced to dissect the hippocampus. Additionally, the anteroposterior and dorsoventral axes are represented. **B|**Grey dashed line shows the hippocampus still attached to the cerebral cortex. **C|** Bold dashed line represents the cut done with the brush to dissect the cerebral cortex after removing the hippocampus, shown in grey dashed line. **D|**Dashed line shows the location of the cut to dissect the cortex. Additionally, the anteroposterior and dorsoventral axes are represented. **E|**Grey dashed line shows the dissected cortex. HP: Hippocampus.

3.4.4 Midbrain dissection

After removal of the hippocampus and the cortex, the remaining brain as the lateroventral side facing the filter paper (**Figure 8A)**. Using the white-coloured structure, which included the pons, part of the medulla and the cerebellum as a reference, two firm parallel cuts were done using the scalpel (**Figure 8A, 7**). While holding the brain with the forceps, the midbrain was smoothly removed using the brush (**Figure 8B**). Thereafter, this procedure was repeated for the other half brain and the dissected tissue was collected in marked Eppendorf tubes and stored in the dry ice.

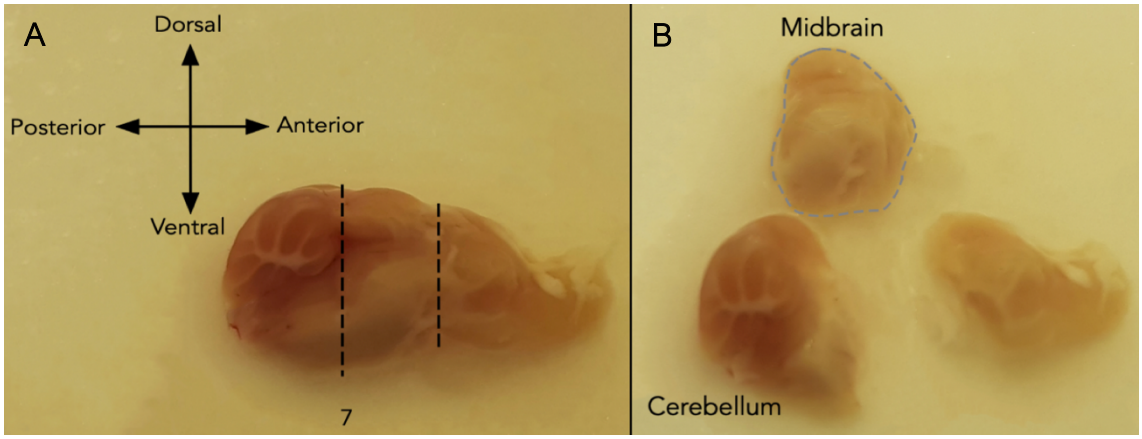


Figure 8 | Dissection of the midbrain. A | Dashed lines show the location of the cuts of midbrain dissection. Additionally, the anteroposterior and dorsoventral axes are represented. **B |** Cerebellum and dissected midbrain are shown.

3.5 Molecular procedures

3.5.1 Bromodeoxyuridine treatment

Cell proliferation and survival *in vivo* can be measured with 5'-bromo-2'-deoxyuridine (BrdU), a thymine analogue that incorporates into the newly synthesized DNA of dividing cells. The incorporation is commonly labelled by immunohistochemical assay using anti-BrdU antibodies (Wojtowicz and Kee, 2006). To assess differently the proliferation and the survival of the new-born cell, BrdU needs to be injected at different concentration and time point. Thus, two cohorts were stablished, where the four experimental groups were included.

Mice were administrated BrdU (Sigma-Aldrich, **table 1**) through intraperitoneal injections. First, BrdU was dissolved in sterile 1X PBS by shaking at 37 ºC for ~ 1 hour. To preserve the quality, dissolved BrdU was stored at -20 ºC until used. Due to light sensitivity, the use of opaque tube was required, and syringes were covered with aluminium foil during injections. On one hand, the survival of new-born neurons was labelled by injecting BrdU at a concentration of 300 mg/ml in PBS four weeks before euthanizing, as neurons are considered mature after four weeks of proliferation (Van Praag et al., 2002). Animals were injected with a single dose as per the body weight. On the other hand, it has been demonstrated that neuronal proliferation can be detected already 24 hours after injection (Wojtowicz and Kee, 2006), thus BrdU was injected four times every two hours at a concentration of 75 mg/ml in PBS, as it provides stronger labelling signal. Although no pain was expected, animals were monitored for their well-being during and after the injection period. Previous experience performing BrdU injections showed mice survive without any side effects on their feeding and social behaviours.

3.5.1.1 BrdU detection by DNA dot-blot

To detect and quantify BrdU incorporation into the DNA we performed a dot-blot method. Although cell proliferation and survival *in vivo* are widely assessed by immunohistochemical methods, these techniques might be tedious and its quantification unreliable (Ueda, 2005). Thus, we decided to combine the immunohistochemical labelling, which results were excluded from this thesis, with a trustworthy and fast technique to quantitate BrdU incorporation in new-born neurons. The DNA dot-blot method has been developed in our laboratory (Wu and Castrén, 2009). In the dot-blot method, we were able to detect the incorporated BrdU using a monoclonal anti-BrdU antibody. Isolated and processed DNA samples were directly applied on a nylon membrane, which was blotted with the valid monoclonal antibody. After the antibody incubation and its corresponding detection method, the quantification was performed using a chemiluminescent substrate (Pierce™ ECL Plus western blotting substrate, **table 1**). The quantification of BrdU was done through the imaging of the membrane and its densitometry analyses using ImageJ (National Institutes of Health, **table 1**).

3.5.1.2 DNA isolation and processing

To isolate the DNA of the hippocampus samples, DNeasy® Blood and Tissue Kit (QIAGEN, **table 1**) was used due to its rapidity and efficacy. Proceeding *as per* the manufacturer's instructions, DNA was extracted from hippocampus samples, which were previously stored at -80 ºC. Upon isolation of the DNA, we used the spectrophotometer NanoDrop 2000_C (ThermoFisher Scientific, **table 1**) to assess the purity of DNA, which is calculated by a modification of the Beer-Lambert equation. When the values were significantly above the extinction coefficient for double-stranded DNA, 50 ng/µl, the DNA extraction was considered as successful. Isolated DNA was kept at -20 ºC until further use.

To enable BrdU detection, the isolated DNA was treated and hybridized with a nylon transfer membrane. First, the isolated double-stranded DNA was converted into single-stranded DNA, which was subsequently hybridized with a positively charged nylon transfer membrane (GE Healthcare Life Science, **table 1**). A modification of the original protocol (Ueda, 2005) was required to reduce the final volume hence, the concentration of the reagents was modified accordingly. Based on the isolated DNA concentration previously calculated, a volume containing 2 µg double-stranded DNA was incubated with one volume of 4 N NaOH solution for 30 minutes at room temperature. The mixture was placed on ice to prevent annealing of the single-stranded DNA. While still on ice, an equal volume of 1M Tris·HCl (pH 6.8) was pipetted to neutralize the solution. To transfer the neutralized single-stranded DNA to the nylon membrane, we used a 96-well micro-sample filtration chamber (Schkeicher and Schuell, **table 1**). The apparatus consists of a 96-well surface incorporated with a vacuum system. Between the 96-well surface and the inner chamber, we placed the nylon membrane, and underneath a cellulose western blotting membrane (GE Healthcare Life Science, **table 1**), which retains the moisture. Then, the 96-well micro-sample filtration chamber was tightly closed to ensure the vacuum. When added the neutralized single-stranded DNA into the well, the pressure produced by the vacuum system rapidly moved the sample from the well to the inner chamber, going through the positively charged nylon membrane. By using this mechanism, we assured that loaded samples did not overlap and that the area of all the samples were equals, which was crucial for further analysis. Finally, the neutralized single-stranded DNA was crosslinked to the nylon membrane using a Stratalalinker® UV

Crosslinker (Stratagene, **table 1**) by selecting the *autocrosslink* mode, which emitted 1200 microjoules (x 100) of UV light for approximately 45 seconds.

3.5.1.3 Immunodetection and densitometry analysis

To assess BrdU incorporation into the DNA of new-born neurons, a chemiluminescent detection method was applied after the nylon membrane was incubated with mouse anti-BrdU monoclonal antibody (Sigma-Aldrich, **table 2**). Once the membrane was crosslinked, it was probed with primary antibody (1:200) in TBS-T containing 1% non-fat dry milk (NFDM) overnight on a rocker at 4 ºC. No blocking buffer incubation was needed to prevent non-specific binding. The following day, the membrane was washed three times for 10 minutes with TBS-T at room temperature. For the detection, the membrane was incubated with a solution containing anti-mouse horseradish peroxidase (HRP) (Bio-Rad, **table 2**) conjugated (1:5000) in 3% NFDM TBS-T for one hour at room temperature. Subsequently, the secondary antibody solution was removed, and the membrane was washed three times for 20 minutes with TBS-T on a rocker at room temperature.

For the quantitative analysis, a chemiluminescent detection method was performed. Nylon membranes were incubated with ECL+ chemiluminescent reagent to enhance the analysis. The oxidation of luminol by the hydrogen peroxidase in the ECL+ emits light, which is detected. Membranes were scanned using LAS3000 camera, which allowed the chemiluminescent detection. Images were taken through Image reader LAS-3000 (Fujifilm, **table 1**), using the increment method in intervals of 30 seconds. The densitometry analysis of the BrdU detection was done using ImageJ Software. Densitometry allowed to create relative comparisons and average values for the relative abundance of BrdU between samples. By manually selecting the images in the membrane, the software generated the densitometry measurements, which were displayed as a graphical representation of the average intensity of pixels. The numerical results were obtained by manually calculating the area of the average intensity graphs. The relative values were obtained through the normalization of the sample's values after subtracting the background, as an equal volume of DNA of each sample was hybridised.

3.5.2 Real-time reverse transcription quantitative polymerase-chain reaction (RT-qPCR)

This molecular technique is based on the reversed transcription of RNA into cDNA and the subsequent amplification of specific DNA targets through polymerase chain reaction (PCR) (Rio, 2014). The amplification reaction can be complemented with fluorescent reactions, which allow the quantification of the target RNA. The technique can be performed in single or two steps. In this study, two-step real-time PCR was used as it allows accurate quantifications.

3.5.2.1 RNA isolation

The RNA extraction and purification were completed using the PureLink® RNA Mini Kit (Ambion® Life technologies, **table 1**), which required extreme aseptic practices such as performing all the procedures under the hood and cleaning the instruments with RNAase decontaminant. Proceeding *as per* the manufacturer's instructions, the RNA was isolated, and the purity was assessed using the Nanodrop 2000_C spectrophotometer, following the protocol instructions previously detailed for DNA purification assessment. Then, RNA was stored at -80 ºC until further use.

Extracted RNA was transcribed into complementary deoxyribonucleic acid strands (cDNA) through reverse transcriptase, which was the first step of the two-step polymerase-chain-reaction. Into a nuclease-free microcentrifuge tube, a mixture of 1 µl of 50 µM oligo$(dT)_{20}$, and 1 µl of 10 mM dNTP Mix was added to a volume of purified RNA containing 5 µg of RNA. Adding nuclease-free water up to 13 µl, the solution was mixed by pipetting and incubated at 65 ºC for five minutes, promoting the denaturation of the RNA, and immediately transferred to ice for ~ two minutes. Afterward, for the cDNA synthesis reaction, 4 µl of 5x first-Strand Buffer and 1 µl of 0.1M DTT were added, which equilibrated the pH of the mixture. To eliminate any impurity, 1 µl RNaseOUT™ (40 units/ µl; ThermoFisher Scientific, **table 1**) was aliquoted, and 1 µl SuperScript™ III RT (200 units/µl; ThermoFisher Scientific, **table 1**) was subsequently added, which allowed the synthesis of the cDNA. The mixture was gently mixed by pipetting. The cDNA synthesis reaction consisted of five minutes incubation at 25 ºC for hexamer primers annealing; followed by 30 minutes incubation at 50 ºC allowing the annealing of

oligo$(dT)_{20}$ primers and the synthesis of cDNA; and finalised by 15 minutes incubation at 70ºC for inactivation of the enzyme mix. The cDNA product, stored at -20 ºC until the subsequent step, was suitable to be used as a template for amplification in PCR.

3.5.2.2 Two-steps real-time PCR and analysis

The two-step real-time PCR was performed with the use of Maxima Hot Start Taq DNA Polymerase (ThermoFisher Scientific, **table 1**), which requires forward and reverse primers of the target gene (**Table 3**) to synthesize a sequence of bases complementary to the single strands of DNA primed. For the real-time PCR reaction, 1 µl (5 µM) of both reverse and forward primer of the target gene, 6 µl of SYBR Green (Fermentas, **table 1**), and 4 µl Nuclease-free water (ThermoFisher Scientific, **table 1**) were pipetted into a white LightCycler® 480 Multiwell Plate 96 (Roche, **table 1**), under extreme aseptic conditions. Thereafter, 1 µl of cDNA (1:20 diluted in Nuclease-free water) were added, reaching a total volume of 12 µl, and the plate was centrifuged and sealed with an optically clear sealing tape. The C1000^{TM} Thermal cycler and the CFX^{TM} Real-time System (Bio-Rad, **table 1**) were used for the real-time PCR reaction, which consisted of 10 minutes at 95 ºC to allow the initial denaturation, followed by 40 cycles of 95 ºC for 30 seconds and 60 ºC for 30 seconds, which permitted the annealing and extension, respectively. The SYBR Green fluorescent DNA probe allowed the detection of polymerase-chain-reaction products through the emission of fluorescent signal, which occurred upon binging to the double-stranded DNA.

Relative gene expression was calculated through the accumulation of fluorescent signal generated in the polymerase-chain-reaction using CFX^{TM} Real-time System. A baseline detection was determined by the CFX^{TM} Real-time Software according to variations in the fluorescence signal during the initial PCR cycles. When in a particular PCR cycle the fluorescent signal exceeded the baseline, C_q was assigned, which was defined as the relative measure of the concentration. The C_q value was inversely proportional to the amount of target nucleic acid of the correspondent gene. In our study, we applied a relative method, which required one housekeeping gene to normalize the results. Thus, the expression of the target gene was normalized with the expression of a housekeeping gene (**Equation 2.1)**. Subsequently, the relative expression of the target gene was easily assessed applying the 2^{-DCq} method (**Equation 2.2**) (Livak and Schmittgen, 2001).

$$(2.1) \qquad \Delta C_q = Target\ Gene\ C_q - Housekeeping\ Gene\ C_q$$

$$(2.2) \qquad Relative\ Gene\ Expression = 2^{-\Delta C_q}$$

Equation 2 | All the calculations were based on the above equations.

3.6 Statistical analysis

In our study, two different housing conditions, EE and SH, were analysed in two groups, control and transgenic mice, resulting in four experimental groups: control-SH, transgenic-SH, control-EE, and transgenic-EE. Before analysing the hypothetical effects of EE in transgenic mice, EE paradigm was validated through the analysis of behavioural battery test results of control mice in SH and EE. Data concerning behavioural test of control and transgenic mice under standard housing conditions acquired previously were compared with the new data obtained and both data were compiled together as no differences were observed. Additionally, as no sex-differences were expected in our transgenic mouse model, male and female data were merged.

All statistical analyses were performed using GraphPad Prism 8® Software (GraphPad Software Inc., **table 1**). To analyse the significance of the comparison of two groups, behavioural tests were analysed either with Student's t-test, or with one-way analysis of variance (ANOVA). This was followed by Tukey's post-hoc test, when the analysis of more than two groups was required. For the analysis of cued and contextual fear conditioning and pre-pulse inhibition of the startle response, in addition to Tukey's multiple comparison test, Sidak's multiple comparison test was performed to analyse differences between the test phases and/or the groups. Moreover, post hoc Bonferroni/Dunn test was performed to reduce false positive, Type I errors. Data were presented as mean ± (SEM), standard error of means. A *p*-value equal to or lower than 0.05 implies the results were statistically significant.

3.7 Reagents

Table 1 | Key resources table.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Behavioural experiments		
Enriched environment cages (90 cm x 90 cm x 32 cm)	University of Helsinki	
IVC500	Tecniplast	GM500
Pipes, plastic toys and running wheels	University of Helsinki	
Aspen bedding material	Tapvei	
Aspen nesting material	Tapvei	
Aspen gnawing s-bricks	Tapvei	
Teklad global 16% protein	Envigo	2016C
Activity Monitor Software	Med Associates, Inc	Version 5.1
Open field arena (30 cm x 30 cm x 30 cm)	Med Associates, Inc	
Dark box (15 cm x 15 cm x 30 cm)	Med Associates, Inc	
Cylindrical Plexiglass 5 litres tank	University of Helsinki	
Novel Object Recognition arena	University of Helsinki	
Startle Reflex System	MED Associates, Inc	Version 5
Fear Conditioning System	TSE System	Version 4.10
EthoVision® XT	Noldus Information Technology	Version 13
Digital camera	Basler AG	106580-08
Sample dissection		
Phosphate-buffered saline (PBS)	University of Helsinki	
RNAse-free 1X PBS	University of Helsinki	

Whatman ® Grade 3MM Chr Cellulose Western blotting membrane	GE Healthcare Life Science	14115086
Student surgical scissors	Fine Science Tools	91402-14
Fine sharp scissors 21mm	Fine Science Tools	14060-10
Hippocampal dissecting tool	Fine Science Tools	10099-15
Graefe serrated Forceps 0.8mm	Fine Science Tools	11051-10
Brush size 2	Hanart oy	
Student Halsted-Mosquito hemostats	Fine Science Tools	91309-12
Bochem chemical spoon 150 mm	Lab Unlimited	4AJ-9150315
Feather disposable scalpel	Feather Safety Razor	5205052
Carbon dioxide solid (dry ice)	Oy AGA Ab	
Tamoxifen injections		
Tamoxifen	Sigma-Aldrich	WXBC1801V
Corn oil	Sigma-Aldrich	MKCC0462
Syringe 1 mL without needle	TERUMO	0301006
Needle 18G x $1^{1/2''}$ 1.2 X 40 mm LUER	TERUMO	1311021
BrdU injections		
Phosphate-buffered saline (PBS)	University of Helsinki	
5-bromo-2'-desoxiurudina	Sigma-Aldrich	HMBG7275V
Syringe 1 mL without needle	TERUMO	0301006
Needle 18G x $1^{1/2''}$ 1.2 X 40 mm LUER	TERUMO	1311021
DNA dot-blot		
DNeasy® Blood and Tissue Kit	QIAGEN	160047454
Syringe 1 mL without needle	TERUMO	0301006
Needle 18G x $1^{1/2''}$ 1.2 X 40 mm LUER	TERUMO	1311021
4N NaOH	University of Helsinki	

1M Tris-HCl (pH 6.8)	University of Helsinki	
MINIFOLD® Microsample filtration SRC096/0	Schkeicher and Schuell, Inc	UF1 8-3
Amersham Hybond™ -N^+	GE Healthcare Life Science	RPN203B
Stratalalinker® UV Crosslinker 2400	Stratagene	400076
Blocking and washing buffer (TBS-T) 20 mM Tris-HCl (pH 7.6) 136 Mm NaCl 0.05% v/v Tween-20 10x TBS	University of Helsinki	
Non-fat dry milk (Skimmed milk powder)	Valio oy	72190137828
Pierce™ ECL Plus Western blotting substrate	ThermoFisher Scientific	TJ270192
RT-qPCR		
PureLink™ RNA Mini Kit	Ambion® Life technologies	1762389
Maxima First Strand cDNA synthesis kit	ThermoFisher Scientific	00676125
SuperScript™ III Reverse Transcriptase (200 U/µl)	ThermoFisher Scientific	1867069
RNaseOUT™ (40 U/µl)	ThermoFisher Scientific	10777-019
Nuclease-free water	ThermoFisher Scientific	00660520
RNAse Away®	Molecular BioProducts	7002
Maxima® SYBER Green/Fluoresc qPCR Master Mix 2X	Fermentas	00079900
LightCycler® 480 Multiwell Plate 96, white	Roche	04729692001
Sealing tape	SARSTEDT	201409-JB
Experimental Models: Organisms/Strains		

Tph2CreERT2 mice	Provided by Prof. Pierre Chambon´s laboratory	
TrkBflox mice	Jackson laboratory	
Software and Algorithms		
Prism 8	GraphPad Software Inc.	Version 8.0.1
LAS-3000	Fujifilm	
Image Reader LAS-3000	Fujifilm	LAS3000IR
Image processing and analysis software - ImageJ	National Institutes of Health	1.50i
Nanodrop™	ThermoFisher Scientific	2000C
CFX Maestro	Bio-Rad	1.0

Table 2|DNA dot-bot antibody and working concentrations.

REAGENT or RESOURCE	**BATCH NUMBER**	**DILUTION**	**SOURCE**	**IDENTIFIER**
Monoclonal anti-BrdU mouse	B2531	1:2000 1% NFDM in TBS-T	Sigma-Aldrich	038M481V
Goat anti-mouse HRP conjugate		1:5000 in 3% NFDM in TBS-T	Bio-Rad	1705047

Table 3|List of primers used.

GENES of INTEREST	**TYPE**	**SEQUENCE**	**SPECIES**	**SUPPLIER**
BDNF-2	Forward	5'-ATTAGCGAGTGGGTCACAGC	Mouse	Sigma-Aldrich
	Reverse	5'-ATTGCGAGTTCCAGTGCCTT	Mouse	Sigma-Aldrich
HDAC6-1	Forward	5'-TTATACGTGTCCCTGCACCG	Mouse	Sigma-Aldrich
	Reverse	5'-TACCTGCATCTCGGCCTACT	Mouse	Sigma-Aldrich
HPRT	Forward	5'-GGGCTTACCTCACTGCTTTCC	Mouse	Sigma-Aldrich
	Reverse	5'-CTAATCACGACGCTGGGACTG	Mouse	Sigma-Aldrich
NR3C1	Forward	5'-GCATGGAGAATTATGACCACGC	Mouse	Sigma-Aldrich
	Reverse	5'-CTGAATCCTGGTATCGCCTTTG	Mouse	Sigma-Aldrich

4. RESULTS

4.1 Environmental enrichment promotes cognitive enhancement and rescues the hyperactive-like phenotype of transgenic mice

To test the putative memory improvement of EE, we performed the novel object recognition (NOR) test described above. The memory was assessed through the discrimination index (DI) value, which reflects the preference of mice to the familiar or novel object, using the frequency of direct interaction, interpreted as a nose poke between mice and the objects. Discrimination value, represented in percentage, was calculated dividing the difference of frequencies between the novel and familiar object by the sum of frequencies of interactions with both objects. Therefore, negative values were interpreted as a preference for the familiar object, whereas positive values showed the preference for the novel object.

The DI showed values slightly inferior to 0% for control and transgenic mice in SH, hence both groups showed either same preference to the novel and familiar object or slightly biased preference to the familiar object (**Figure 9A**). Under EE conditions, however, significant increments for the novel object were observed in both the groups, reporting a shift of preference (p-value < 0.0001; **Figure 9A**).

Considering the ambulatory distance (cm), the total distance walked during the test session, a significant difference was observed between control and transgenic in SH, where transgenic mice travelled approximately one third more than control mice (p-value < 0.0001; **Figure 9B**). In contrast, roughly similar values of ambulatory distance (cm) were observed in EE, where no significant difference was observed between genotypes (**Figure 9B**).

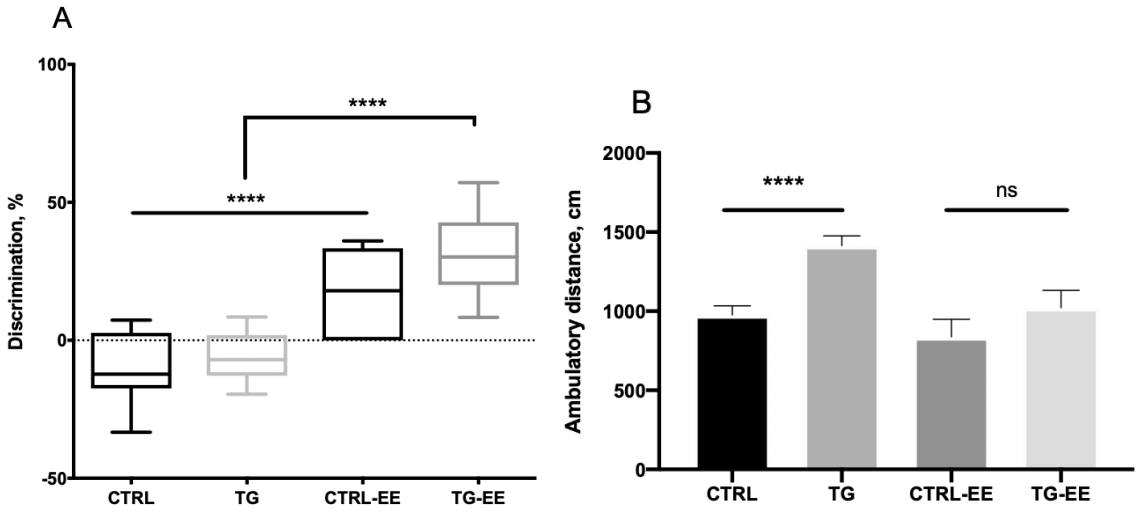


Figure 9 | Memory task improvement and reduction of the hyperactive-like phenotype of transgenic mice subjected to EE. A | NOR test showed a significant shift of the discrimination (%) in both groups after 7 weeks of EE. **B**. The ambulatory distance was reduced in EE conditions, which erased the phenotypical differences that were observed between control and transgenic mice in SH. ****$p < 0.0001$. Significance was tested with one-way ANOVA Tukey's multiple comparison test, assuming samples with equal variation. All data were given as mean ± SEM: $n = 35 - 37$ for each CTRL and TG group; $n = 11 - 12$ for each CTRL-EE and TG-EE group.

4.2 Enriched environment promotes anxiolytic and antidepressant-like effects

Amongst the most robust induced effects of EE are the anxiolytic and anti-depressive-like effects, which can be assessed through diverse behavioural tests. We performed the light-dark (LD) test to evaluate the effects of EE paradigm. The ambulatory distance (cm) in the light compartment, which is an indicator of anxiolytic-like behaviour, showed a significant difference between control and transgenic mice in SH (p-value = 0.0286; **Figure 10A**). Significant increments of the ambulatory distance (cm) were observed in both groups control and transgenic in EE compared to SH, p-value = 0.004 and p-value = 0.0085 respectively, denoting similar effect of EE regardless the genotype. As a result of this generalize increment, no difference was observed between groups in EE (**Figure 10A**). Interestingly, no changes were observed in the ambulatory distance (cm) in the dark compartment between genotypes or housing conditions (**Figure 10B**).

Furthermore, to observe a shift in the compartment preference, we analysed the time spent in light, which was defined as the percentage of the time mice spent in the light compartment divided by the total session time. In SH conditions, control and transgenic

mice showed a significant difference in the time spent in the light compartment (p-value = 0.0265), whereas no differences were observed under EE conditions (**Figure 10C**). EE significantly raised the time spent in light (%) in both control and transgenic mice, p-value = 0.0007 and p-value = 0.00267 respectively (**Figure 10C**). Thus, the time spent in the light compartment in control mice increased by approximately 25% whereas EE raised the time spent in light from roughly 25% to almost 40%.

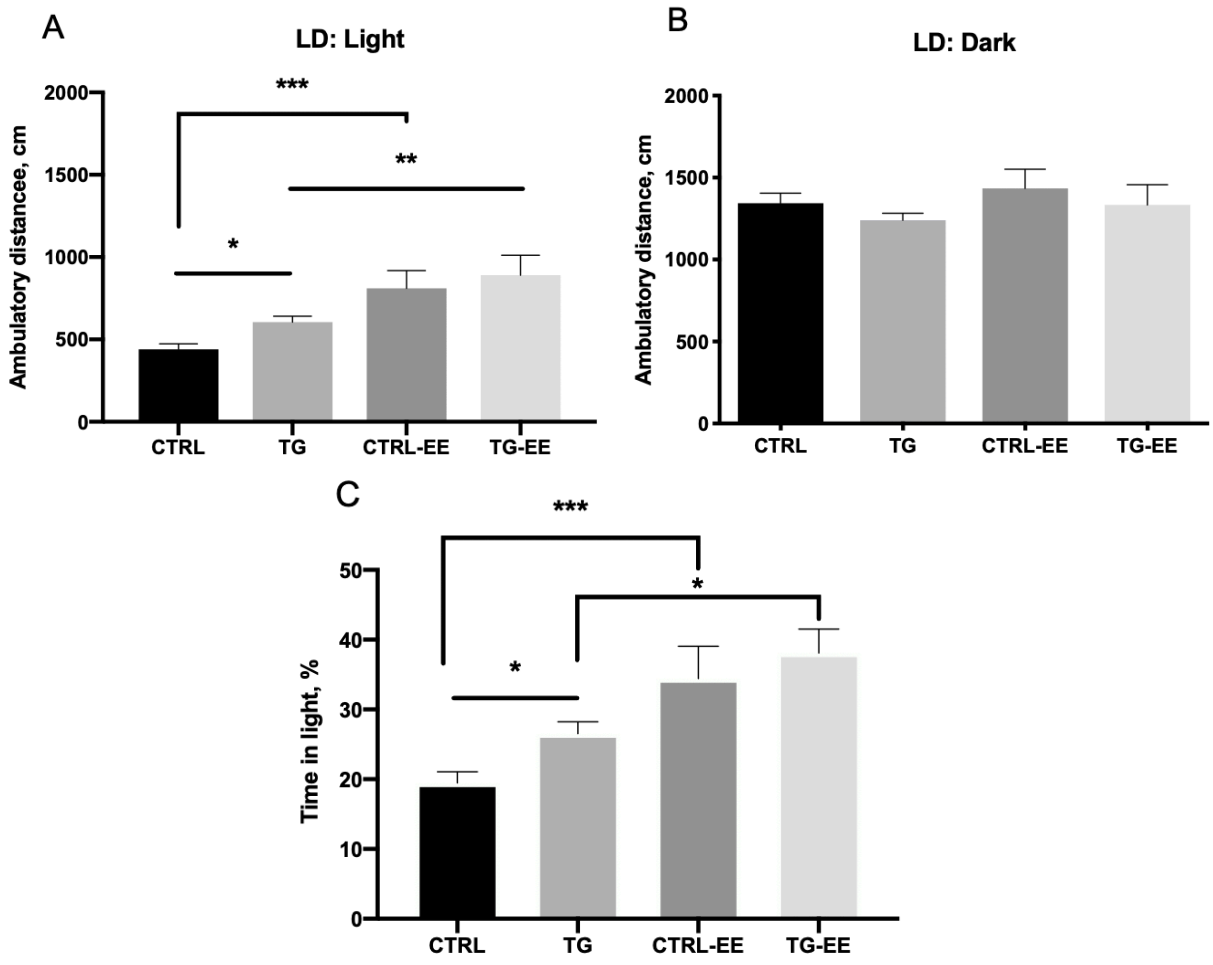


Figure 10|Anxiolytic-like effects reported upon EE housing conditions. A|LD test revealed a significant increment of the ambulatory distance in the light compartment for both control and transgenic mice. EE also erased the phenotypical difference observed in SH between control and transgenic mice. **B|**No statistically significant changes were observed in the ambulatory distance in the dark compartment. **C|**The percentage of time spent in the light was significantly increased upon EE. Additionally, EE eliminated that phenotypical differences observed between control and transgenic group in SH conditions. *** $p < 0.0002$; **$p < 0.005$; *$p < 0.05$. Significance was tested with one-way ANOVA Tukey's multiple comparison test, assuming samples with equal variation. All data were given as mean ± SEM: n = 35 - 37 for each CTRL and TG group; n = 10 - 11 for each CTRL-EE and TG-EE group. Data are representative of two independent experiments.

As a non-aversive test to assess anxiety or obsessive-compulsive disorder, we performed marble burying test. Combined with LD, the marble burying test was aimed to indicate the putative anxiolytic-like effects of EE paradigm. The percentage of buried marbles was similar between all the groups at 5 minutes, and despite slight differences at 10 minutes, a similar burying behaviour was reported at 15 minutes (**Figure 11**). The experiment was completed with a similar percentage of buried marbles in all the group.

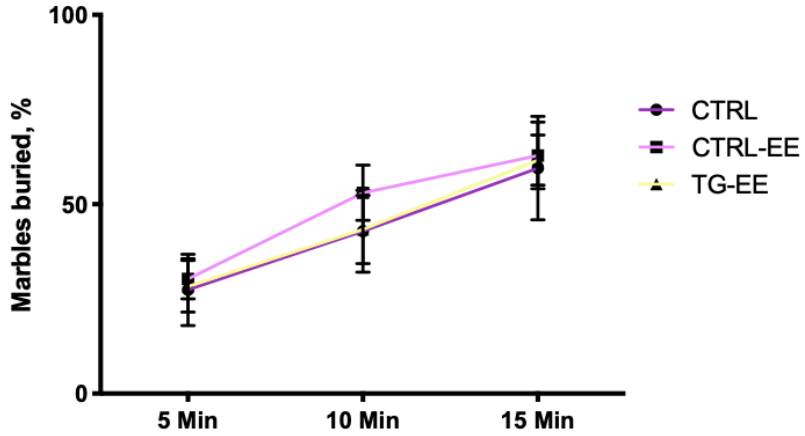


Figure 11|Similar burying behaviours were reported, revealing no anxiety or obsessive-compulsive disorder. Marbles were progressively buried regardless of the housing conditions or the genotype. Data collected manually. Data regarding TG group could not be collected. Significance was tested with one-way ANOVA Tukey's multiple comparison test (assuming samples with equal variation). All data were given as mean ± SEM: n = 8 - 11 per group.

As described in the literature, anti-depressive-like effects can be induced in EE-living conditions. We performed the forced swimming test (FST) to evaluate the putative outcomes of our EE paradigm. For this purpose, the immobility time (s), defined as the time mice spent trying not to escape, was considered as the main parameter to assess anti-depressive-like effects. In SH conditions, transgenic mice showed slightly higher immobility time values compared to control mice. Conversely, this trend was opposite in EE, where transgenic mice showed lower immobility time compared to control mice (**Figure 12**). Interestingly, EE-living conditions only reduced the immobility time in transgenic mice (p-value = 0.0349; **Figure 12**).

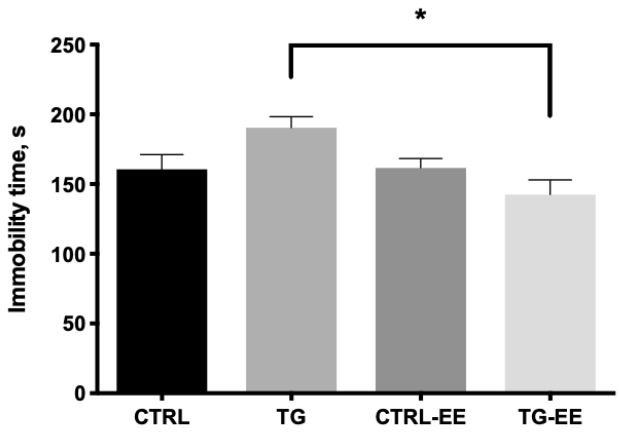


Figure 12 | Immobility time was reduced only in the transgenic mice subjected to EE. FST indicated no effects of EE in control mice, whereas a significant reduction of the immobility time was observed in transgenic mice upon EE. *$p < 0.05$. Data analysed manually. Significance was tested with one-way ANOVA Tukey's multiple comparison test, assuming samples with equal variation. Data were given as mean ± SEM: $n = 9$ -11 per group.

To assess the anxiolytic-like effects we performed the pre-pulse inhibition of the startle response (PPI) test. By measuring the amplitude of the startle response to the 120-dB startle stimulus when emitted without or with pre-pulse stimulus, the anxiolytic-like effects could be measured. When the startle response to the 120-dB startle stimulus was observed throughout the experiment, we observed significant differences between control and transgenic mice in SH in the first two blocks of the experiment and similar trend in the third block (p-value = 0.0206 and p-value = 0.0302; **Figure 13A**). Therefore, transgenic mice showed significantly higher levels of startle amplitude compared to control mice. In contrast, these differences were reduced in EE, where no significant differences between control and transgenic mice were reported. Thus, EE showed a normalization of the startle response between genotype by significantly reducing the startle amplitude of transgenic mice in the three experimental blocks (p-value = 0.0107, p-value = 0.0050, and p-value = 0.0071; **Figure 13A**).

The percentage of pre-pulse inhibition (%), as described previously was used as an anxiolytic-like effects indicator. By analysing the pre-pulse inhibition (%) depending on the different pre-pulse stimuli intensities, no significant differences between the groups were observed (**Figure 13B)**. Regardless of the genotype or living conditions, higher pre-pulse stimulus intensities induced bigger pre-pulse inhibition (%), which showed a direct

relation between the intensity of the pre-pulse and the subsequent pre-pulse inhibition (%), as formerly described in the literature.

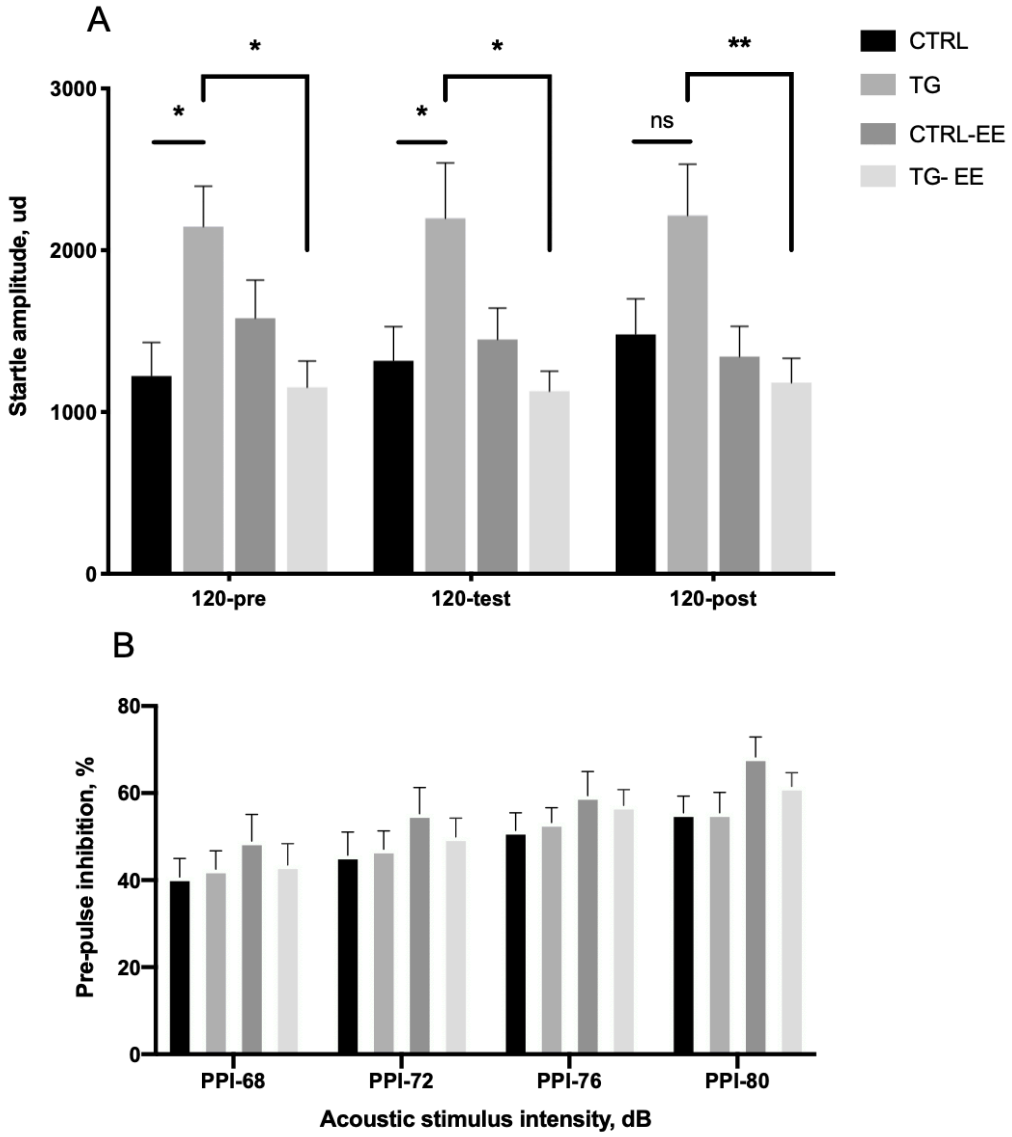


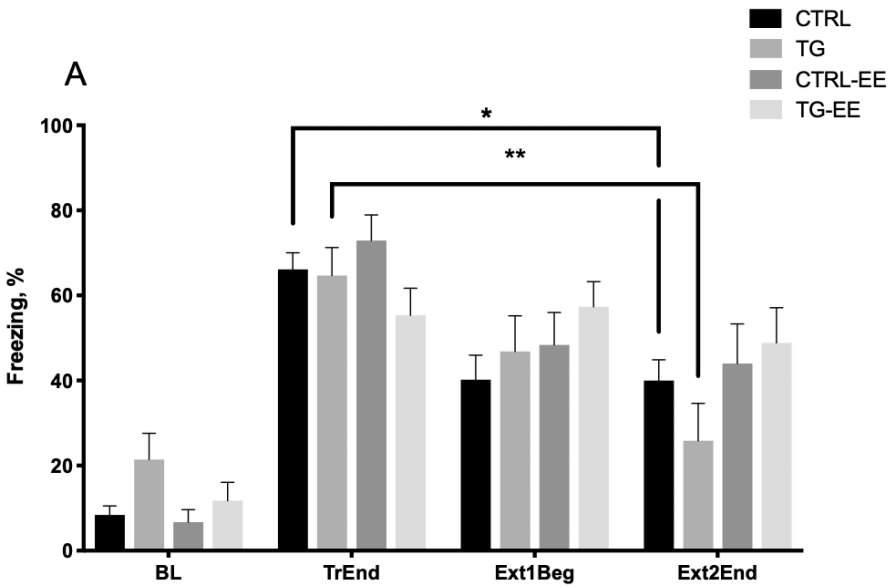
Figure 13|Reduction of the startle amplitude and hence, increase of pre-pulse inhibition was observed upon EE, suggesting anxiolytic-like effects. A|The average of the startle response amplitude to 120-dB tone was represented for: initial five trials of block-1 (120-pre), during the testing trials of block-2 (120-test) and five final trials of block-3 (120-post). EE significantly reduced the startle amplitude of transgenic mice and normalized the response of control and transgenic mice. **B|**The average of pre-pulse inhibition (%) is showed for the four acoustic pre-pulse stimulus intensities used (dB): 68, 72, 76 and 80. Increment of pre-pulse intensity triggered stronger pre-pulse inhibitions (%)**.** $^{**}p < 0.005$; $^{*}p < 0.05$. Significance was tested with two-way ANOVA Tukey's multiple comparison test, assuming samples with equal variation, while two-way ANOVA Sidak's multiple comparisons test was used to assess the differences between

group within the experimental phases. Data were given as mean ± SEM: n = 16 -18 per group. Data were representative of two independent experiments.

4.3 Environmental enrichment increases the freezing behaviour and impairs fear extinction

The cued and contextual fear conditioning (FC) test was performed to assess the freezing behaviour of mice as an indicator of the associative memory of fear-evoking contexts and how EE could have affected that. Similar baseline freezing behaviour (BL) and fear acquisition (TrEnd) were observed regardless of the living conditions and genotype (**Figure 14A**). Throughout the fear extinction sessions, control and transgenic mice under SH showed a progressive reduction of freezing levels, which archived significant values at the end of the second session (p-value = 0.0397 and p-value = 0.0047, respectively). Thus, fear extinction was accomplished in SH living conditions. Curiously, a general increment of freezing behaviour was reported upon EE in both control and transgenic mice, leading to an impartment of fear extinction (**Figure 14A**).

Seven days after the last fear extinction phase, fear renewal was performed by placing the animals in the conditioning context A, as previously detailed under methods section. No statistically significant differences were observed in the freezing behaviour between the acquisition phase (TrEnd) and renewal phase (Ren) (**Figure 14B**). Thus, indicating that control and transgenic mice under SH conditions were able to remember the conditioning context A after achieving fear extinction. Data regarding EE mice were not included in this graphic since EE impaired fear extinction, as previously mentioned.



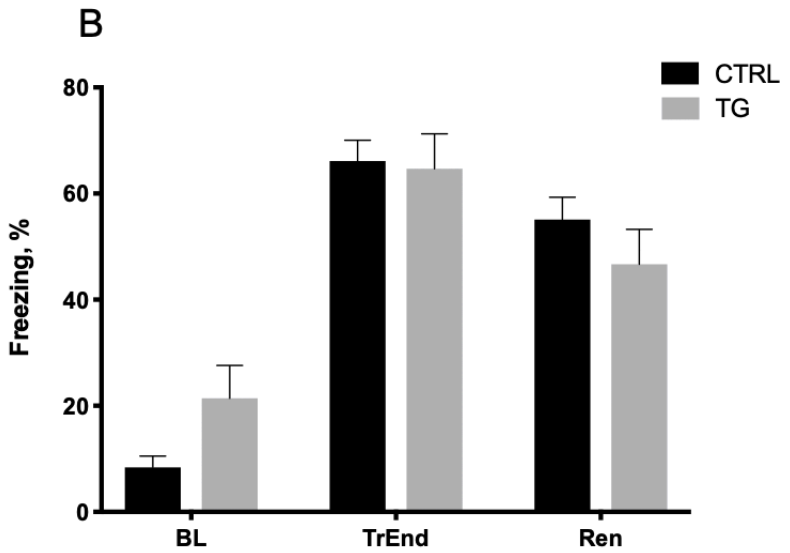


Figure 14|Enriched environment impairs fear erasure when combined with extinction training. A| No statistically significant changes were observed neither in the baseline freezing (BL) behaviour nor in the levels of fear acquisition (TrEnd). Upon SH conditions, control and transgenic mice froze less, thus fear extinction was accomplished at the end of the extinction phase 2 (Ext2End). However, increase in the freezing behaviour of control and transgenic mice were observed in EE living conditions. **B|** Fear renewal was observed in both groups control and transgenic mice upon SH. Similar freezing levels were reported in fear acquisition and fear renewal phase for both groups. Mean of freezing time represented by each experimental phase for control and transgenic mice. Significance was tested with two-way ANOVA Tukey's multiple comparison test, assuming samples with equal variation, while two-way ANOVA Sidak's multiple comparisons test was used to assess the differences between groups within the experimental phases. $^{**}p < 0.005$; $^{*}p < 0.05$. Data were given as mean ± SEM: n = 12 - 15 per group. Data were representative of two independent experiments. BL: baseline; TrEnd: the end of the training phase; Ext1Beg: the beginning of the extinction 1; ExtEnd: the end of the extinction 2; Ren: Renewal

4.4 Environmental enrichment ameliorates the abnormal regulation of adult hippocampal neurogenesis of transgenic mice

To study the effect of EE on AHN when BDNF-TrkB signalling is compromised from serotonergic neurons, BrdU was intraperitoneally injected to mice of both groups and its incorporation was quantified through the DNA dot-blot method, as detailed under Methods and Materials. Through this method, the proliferation of NSCs in the hippocampus was observed as the relative BrdU incorporation (ud). In SH conditions, the hippocampal proliferation of NSCs of transgenic mice was roughly 65% higher than the proliferation detected in control mice (*p*-value = 0.00170; **Figure 15A**). This difference,

contrastingly, was no reported between groups in EE (**Figure 15B**). Upon EE, therefore, a normalization of the AHN regulation occurred, and hippocampal proliferation of NSCs showed similar values.

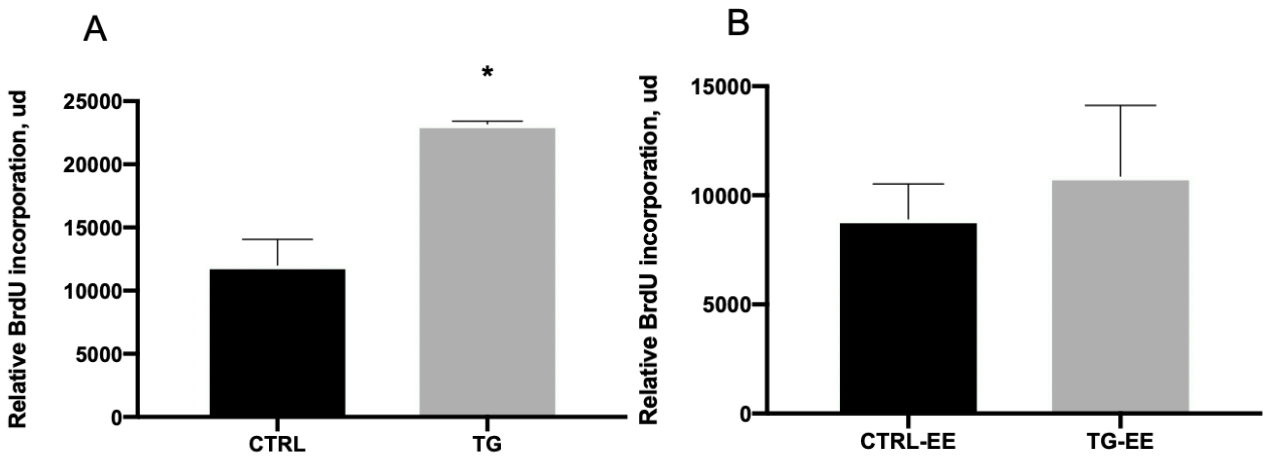


Figure 15 | Proliferation of hippocampal granule cells was normalized upon EE. A-B | Hippocampal tissue samples were proceeded and the relative BrdU incorporation (ud) was assessed through densitometric analysis of the detection of mAB anti-BrdU. Whereas the proliferation of NSCs showed a significant difference in SH conditions **(A)**, the difference was reduced in EE-living conditions **(B).** *$p < 0.05$. Significance was tested with unpaired *t*-test. All data were given as mean ± SEM: $n = 3$ -5 per group.

4.5 Environmental enrichment affects epigenetic modification observed in the hippocampus

To depict the influence of long-term EE-housing conditions on the neuronal gene expression, we performed qRT-PCR. This technique allowed the analysis of the putative changes in the gene expression of genes closely related to neuronal epigenetic modifications, such as *Bdnf,* due to its crucial role in cell differentiation and survival; the glucocorticoid receptor (*Nr3c1*), resulting from its role as a transcription factor related to cellular proliferation and differentiation; and the *histone deacetylase 6* (*Hdac6*), due to its regulation in the serotonin pathways implicated in the stress-related behaviours (Mouse Genome Database 2019). Normalization of gene expression was performed against the house-keeping gene *hypoxanthine phosphoribosyltransferase* 1 (*Hprt 1*), which encoded for a transferase with a crucial role in the generation of purine nucleotides (Mouse Genome Database 2019).

We observed an upregulation of roughly 50% of the *Bdnf* expression in control and transgenic groups after EE conditions, being the increase significant in transgenic mice (*p*-value = 0.0208; **Figure 16A**). Likewise, noticeable enhancements were reported in *Nr3c1* expression, which was significantly increased in both control and transgenic mice upon EE (*p*-value = 0.0580, *p*-value = 0.0153 respectively; **Figure 16B**). The expression of *Hdac6* was similarly upregulated in both control and transgenic mice in EE. The increase was significant exclusively in transgenic mice (*p*-value = 0.0071; **Figure 16C**). Altogether, a general increment of the gene expression and normalization of the differences between genotypes were observed after EE-living conditions.

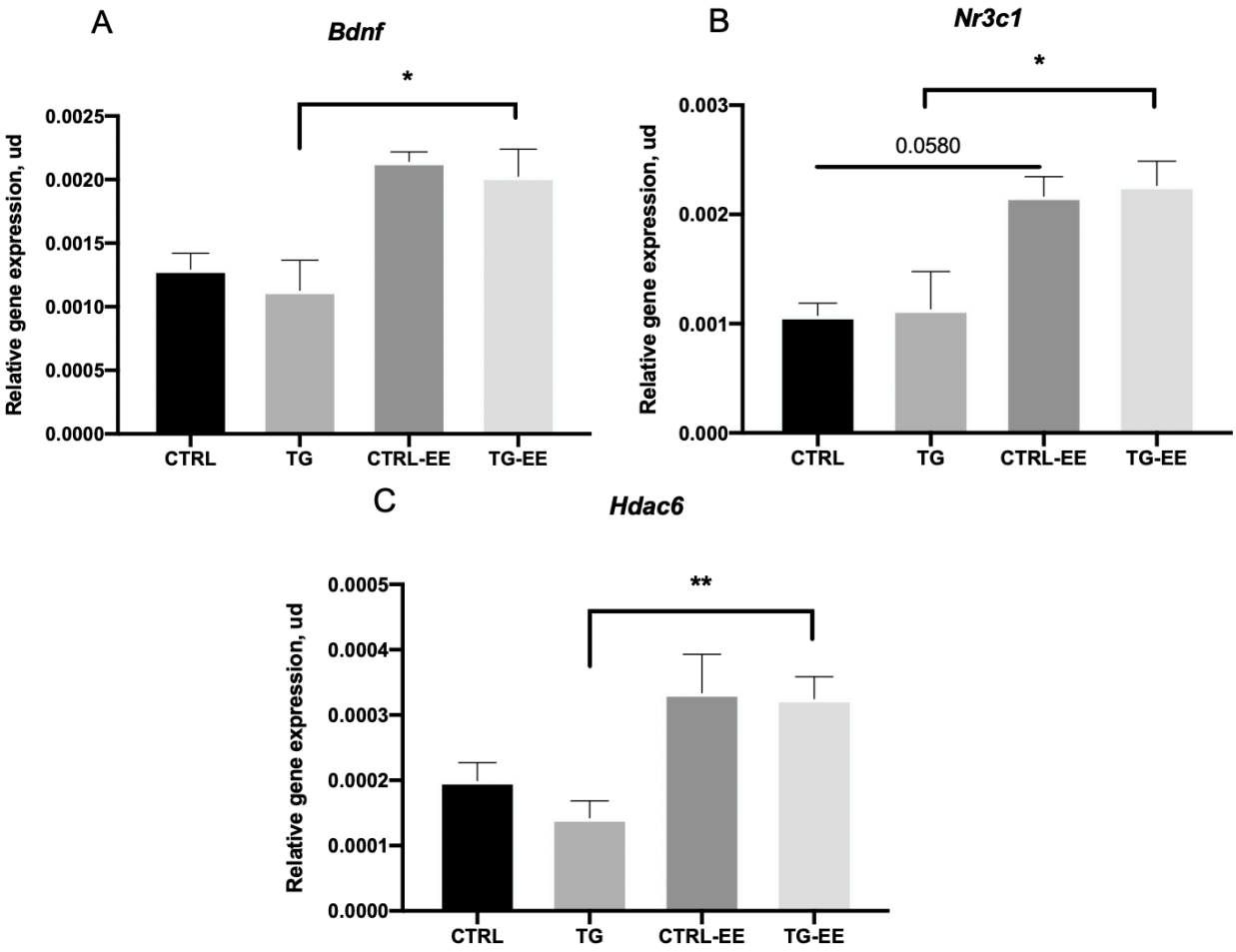


Figure 16 | Regulation of the gene expression involved in cell proliferation, differentiation and epigenetic modification upon EE. A | Measured by qRT-PCR, *Bdnf* gene expression was increased upon EE in control and transgenic group. **B |** Significant changes were observed in the upregulation of *Nr3c1* gene in EE regardless of the experimental group. **C.** EE upregulated the expression of *Hdac6*, reducing also the differences between control and transgenic groups. $^{**}p < 0.005$; $^{*}p < 0.05$. Significance was tested with one-way ANOVA Tukey's multiple comparison test, assuming samples with equal variation. All data were given as mean ± SEM: $n = 4$ - 6 per group.

5. DISCUSSION

The main objective of this thesis was to study the behavioural and neurogenic effects of EE in a conditional knockout mice line whereby BDNF-TrkB signalling is compromised from the serotonergic neurons. In order to assess the effects of EE paradigm, we performed a behavioural battery of tests whereby memory improvement, anxiolytic and antidepressant-like effect were reported. Next, to investigate the effects of EE on AHN, animals were injected with BrdU and the analysis revealed a controlled regulation of AHN. Finally, we determined an EE modulation of the expression of crucial genes related to plasticity together with genes of epigenetic modifications.

Our first goal was to determine the effectiveness of EE-living conditions in our knockout animals, so we performed a behavioural battery of test to assess memory and learning together with anxiolytic- and antidepressant-like effects. Significant memory improvements were reported through NOR test, whereby an evident shift of object preference was observed regardless of the phenotype (Viola et al. 2010; Mesa-Gresa, Pérez-Martinez, and Redolat 2013). While clear cognitive benefits were distinctly reported as a consequence of EE in NOR, our results obtained in FC test were unclear. EE significantly increased freezing behaviour throughout the experiment regardless of the phenotype hence, fear renewal results were difficult to interpret. While extinction was achieved in SH for both control and transgenic mice, renewal was deficient in the transgenic mice. In agreement with previous findings, the timing and length of EE paradigm play a crucial role in the extinction of fear memory, which can be affected by a non-associative factor such context generalization (Hegde, O'Mara, and Laxmi, 2017).

Anxiolytic and antidepressant-like effects were also clearly observed as a consequence of EE-living conditions. In LD, we detected the increased preference for bright, open spaces regardless of the phenotype, suggesting anxiolytic behaviour. These effects have been thoroughly reported as a clear benefit of EE-living conditions (Roy et al. 2001; Leger et al. 2015). In PPI, furthermore, we reported a significant reduction of innate anxiety as drastic decrease of startle amplitude. Our transgenic mice showed an excessive startle response baseline to acoustic stimuli, which suggests sensorimotor gating impairment. Upon EE, startle response results normalized and no difference was observed between the genotypes. Strikingly, a recent study postulated that three-weeks EE-living conditions

in a knockout mice with low baseline startle response showed no effect on PPI (Rogers, Li, Lanfumey, Hannan, and Renoir, 2017). Comparing these results with ours, we could conclude that the length of EE together with the sensorimotor gating performance of the knockout are both two relevant factors to take into consideration when using PPI to assess EE effects. Instead, antidepressant-like effects were reported exclusively in transgenic mice in FST, measured as a decrease of immobility time (s). The antidepressant-like effects observed solely in transgenic mice upon EE differs from unpublished, previous data from our lab. The previous results showed no effect of SRRI fluoxetine acute treatment on our transgenic mice, while EE has a significant effect. Further experiments are currently carried out to compare the underlying molecular mechanisms of EE and classical antidepressants. These studies could potentially unfold possibilities for combinatorial treatments in treatment-resistant cases.

The hyperactive-like phenotype of transgenic mice, expressed as hyper-locomotor activity, was attenuated as a consequence of EE-living conditions. This conclusion was derived from NOR and LD results. Significant differences observed in ambulatory distance between control and transgenic mice were reduced, indicating a normalization of the hyperactive-like phenotype as a direct consequence of EE-living conditions. In conformity with our results, previous studies have demonstrated that EE rescues hyperactivity and impulsivity in rodent models. This is mediated through EE-dependent plasticity, which indicates the potential therapeutic uses to treat neurological disorders (Alwis and Rajan 2014; Botanas et al. 2016).

Next, we aimed to analyse the neuronal effects of EE on the regulation of AHN as a putative driver in accordance with the behavioural battery test. For this purpose, BrdU was injected into animals to detect cell proliferation and survival. This was assessed by the DNA dot-blot technique and densiometric analysis. Relative BrdU incorporation values from hippocampus showed prominent cell proliferation of transgenic mice compared to control. Previous results in these mice demonstrate, the abnormal regulation of AHN leading to aberrant cell proliferation. When subjected to EE, no significant difference between groups to relative BrdU incorporation levels of cell proliferation was observed. Thus, an improvement of the abnormal regulation of AHN is observed by EE-living conditions even with a genetic defect.

Surprisingly, we also reported a slight decrease of proliferation in control mice upon EE, in contrast with previous studies which have demonstrated that EE, especially through voluntary exercise, promotes cell proliferation (Kempermann, Kuhn, and Gage 1997; Van Praag, Kempermann, and Gage 1999; Kempermann et al. 2018). We hypothesise that this scarce diminution of relative BrdU incorporation values could be explained by a deficient standardization of hippocampal dissection and collection of the sample. As previously detailed under the methods section, the entire hippocampus was dissected and proceeded for the study of AHN through the analysis of BrdU incorporation. Recent findings have demonstrated AHN is regulated by EE differentially along the dorsoventral axis, which it reflects as differential proliferation depending on the area of the hippocampus (Zheng et al. 2017; Zhang et al. 2018). To check the cell survival of new-born neurons caused by the rescue of AHN regulation, we studied the relative BrdU incorporation by injecting the animals four weeks before termination. However, no results were obtained of BrdU detection of cell survival of any experimental group. As even positive controls were not able to be detected, we hypothesise that BrdU detection of cell survival by DNA dot-blot method could have presented unappropriated detection limits. Therefore, we hypothesize that our results may be explained by the lack of dorsoventral axis' differentiation while dissecting the hippocampus. This dorsoventral asymmetry needs to be considered when studying AHN. Although this technique may be suitable to detect cell survival in the tissue of rapid turnover in peripheral organs, the detection in hippocampus tissue may result difficult due to the low-rate of neurons' turnover (Ueda 2005). Besides, cell survival detection of new-born neurons needs to be performed four weeks after the BrdU injections to ensure the achievement of the survival stage, the effect gets undetected. This time-requirement may directly affect the DNA dot-blot method's detection outcomes. Alternatively, the immunohistochemistry study of perfused brain samples is currently being performed and we aim to confirm the promotion of cell survival through the labelling of cell survival markers.

To understand the effects of EE on AHN and depict the underlying mechanism, we performed qRT-PCR to analyse the influence of EE on the neuronal gene expression of genes closely related with the mechanism of neuronal plasticity and epigenetic modifications. EE prominently upregulated the expression of *Bdnf* and *Nr3c1*, two key players in cellular survival and differentiation, respectively. Additionally, we observed significant increment in *Hdac6* expression, a driver of epigenetic modifications. Although

the results are exclusively a preliminary screening, we obtained consistent data to speculate that EE could promote the survival of the neurons by epigenetic modifications. Further studies such as a high-throughput RNA-seq could provide more evidence of how the EE regulates the phenotype of our conditional knockout mice.

To summarize, we provided evidences that 10-weeks EE-living conditions result in reduced hyperactive-like behaviour, enhancement of learning tasks and induced anxiolytic and antidepressant-like effects. We also proved the compensatory effect of EE on the impaired AHN regulation of knockout mice. Further analysis should be continued to determine whether EE is promoting the survival of new-born neurons. Our preliminary results revealed altered expression of genes closely related to neuronal plasticity and epigenetic modifications. These findings emphasize the therapeutic effects of EE and the significance of understanding adult brain plasticity and its underlying mechanisms. This could to depict antidepressant mechanisms and establish combinatorial therapy for treatment-resistant patients.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Considering the results obtained in this thesis, we are able to conclude that:

1. Consistent neurological effects are induced upon EE in line with previous publications.

2. Reduction of hyper-locomotor and hyperactive behaviour in transgenic mice subjected to EE.

3. EE rescues the abnormal regulation of AHN, and it compensates the lack of TrkB in serotonergic modulation of AHN.

4. Upregulation of important genes related to cell proliferation and epigenetic modifications in animals subjected to EE.

In the future experiments, it would be necessary to re-examine cell survival both through immunohistochemistry method and an alternative technique such as the study of immediate early genes detecting neuronal activity markers. Furthermore, to gain a better understanding of the underlying mechanisms involved, the differential expression of serotonergic receptors and other epigenetic markers as putative drivers of the plasticity mediated mechanisms should be explored. Due to the remarkable effects of EE in our transgenic mice model, it would be very interesting to further study the effects of EE paradigm on other brain areas such as ocular dominance plasticity in the visual cortex and also in other conditional knockouts. Finally, it would be suitable to compare the effects with classical antidepressants and/or the combination of both.

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